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(54) Title: MCP-3, RANTES AND MIP-1ALPHA RECEPTOR ANTAGONISTS (57) Abstract The present invention is directed towards NH ₂ -terminally truncated analogs of three human chemokines: MCP-3, RANTES and MIP-1 α having highly potent anti-inflammatory activity and anti-autoimmune activity. The present invention is also directed to inhibiting the biological activities of three native, mammalian chemokines: MCP-3, RANTES and MIP-1 α . The present invention is further directed to treating inflammatory diseases and autoimmune disorders such as rheumatoid arthritis, for example. The present invention is also directed to pharmaceutical compositions comprising NH ₂ -terminally truncated chemokine analogs.		

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MCP-3, RANTES AND MIP-1ALPHA RECEPTOR ANTAGONISTS

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GOVERNMENT SPONSORSHIP

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FIELD OF THE INVENTION

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This invention relates to NH₂-terminally
truncated analogs of Monocyte Chemoattractant Protein-
3 (MCP-3), RANTES and Macrophage Inflammatory Protein-
1α (MIP-1α) and compositions containing same and
methods of employing said compositions for treating
inflammatory diseases as well as autoimmune disorders.

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BACKGROUND OF THE INVENTION

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Regulated on Activation, Normal T-Cell
Expressed and Secreted (RANTES), Monocyte
Chemoattractant Protein-3 (MCP-3) and Macrophage
Inflammatory Protein-1α (MIP-1α) are inflammatory
mediators, characterized as chemotactic cytokines or
chemokines. The term "chemokine" reflects the ability
of these mediators to induce directed migration of
several types of leukocytes, including monocytes,
lymphocytes, basophils and eosinophils into sites of
inflammation.

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RANTES, MCP-3 and MIP-1α are collectively
known as "CC" chemokines because the first two
cysteines in each molecule are adjacent. The CC

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chemokines contain disulfide bridges. The CC
1 chemokines have been implicated in a number of
allergic and chronic inflammatory diseases such as
arthritis and various lung diseases. In such
conditions, early monocytic infiltration of monocytes,
5 T-lymphocytes and other leukocytes may be a key event
in the progression of the disease.

Multiple receptors mediate the functional
activities of the CC chemokines. Five receptors have
been identified and their polypeptide sequences
10 deduced from cDNA clones. These are: chemokine
receptor CCKR-1, which binds MIP-1 α , RANTES and MCP-3
(Gao, et al. (1995) J. Exp. Med. 177:1421-1427); CCKR-
2, which binds MCP-3 and MCP-1 (Franci, et al. (1995)
J. Immunol. 154:6511-6517); CCKR-3, which binds to
15 RANTES, MCP-3 and Eotaxin (Kitaura, et al. (1996) J.
Biol. Chem. 271:7725-7730); CCKR₄, which binds to MCP-
1, RANTES and MIP-1 α (Hoogawerf, et al. (1996)
Biochem. Biophys. Research Communication 218:337-343),
CCKR₅, which binds to MIP-1 α / β and RANTES (Raport, et
20 al. (1996) J. Biol. Chem. 271:17161-17166).

The complete amino acid sequences of RANTES,
MCP-3 and MIP-1 α were described in Clark-Lewis, et al.
(1995) J. Leukocyte Biol. 57:703-711. RANTES
comprises a 68 amino acid polypeptide, MCP-3 comprises
25 a 76 amino acid polypeptide and MIP-1 α comprises a 70
amino acid polypeptide.

It has been suggested that substances that
are capable of blocking the effects of RANTES, MCP-3
and/or MIP-1 α would be useful to moderate or inhibit
30 various inflammatory and allergy reactions. The

patent application of Wells, et al. filed under the
1 Patent Cooperation Treaty and published on June 13,
1996 under WO 96/17935, describes modified RANTES
polypeptides possessing antagonistic activity. The
polypeptides of Wells, et al. comprise the addition of
5 amino acids to the N-terminal end of RANTES so that
the number of amino acids in the antagonists described
therein have a longer chain length than the
corresponding native RANTES.

Masure, et al. (1995) J. Interferon and
10 Cytokine Res. 15:955-963, describe the expression of
mutant MCP-3 in Pichia pastoris which possesses three
additional NH₂ terminal amino acids. Masure, et al.
characterized mutant MCP-3 as an MCP-3 receptor
antagonist capable of competing with synthetic MCP-3.

15 Heretofore, no one knew that NH₂ terminally
truncated chemokine analogs of RANTES, MCP-3 or MIP-1 α
possessed significant antagonistic activity with very
high receptor binding affinities. However, the
present inventors have not only discovered the
20 antagonistic effects of truncated RANTES, MCP-3 and
MIP-1 α , but also have discovered that the analogs are
useful for treating inflammatory diseases and
autoimmune disorders, e.g., rheumatoid arthritis. The
inventors have also discovered that the analogs
25 competitively bind and cross-bind to several different
receptors. Inflammation involves the infiltration of
multiple cell types that occurs through the
interaction of different chemokines with distinct
functional receptors. The inventors have further
30 discovered that blocking the infiltration of multiple

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effector cells is highly effective in breaking the
1 inflammation and autoimmune cycle.

SUMMARY OF THE INVENTION

5 The present invention is directed towards
NH₂-terminally truncated analogs of three human
chemokines: MCP-3, RANTES and MIP-1 α having anti-
inflammatory activity and/or anti-autoimmune activity.
The present invention is also directed to inhibiting
10 the biological activities of three native, mammalian
chemokines MCP-3, RANTES and MIP-1 α . The present
invention is further directed to treating inflammatory
diseases and autoimmune disorders such as rheumatoid
arthritis and multiple sclerosis, for example. The
15 present invention is also directed to pharmaceutical
compositions comprising NH₂-terminally truncated
chemokine analogs.

One aspect of the present invention is
directed to an analog of mammalian MCP-3 lacking NH₂-
20 terminal amino acids corresponding to amino acid
residues 1-6, 1-7, 1-8, 1-9 or 1-10, of MCP-3, having
substantial homology to the native MCP-3 molecule.

Another aspect of the present invention is
directed to an analog of mammalian RANTES lacking NH₂-
25 terminal amino acids corresponding to amino acid
residues 1-5, 1-6, 1-7, 1-8, and 1-9 of RANTES, having
substantial homology to the native RANTES molecule.

A further aspect of the present invention is
directed to an analog of mammalian MIP-1 α lacking NH₂-
30 terminal amino acids corresponding to amino acid

residues 1-9 or 1-10, having substantial homology to
1 the native MIP-1 α molecule.

A still further aspect of the present invention is directed to a method of inhibiting the biological activity or the in vivo biological activity
5 of native MCP-3, RANTES and MIP-1 α comprising adding to the native MCP-3 RANTES and MIP-1 α (if in vitro) or if in vivo administering to a host, e.g., mammal (for example, human) a therapeutically effective amount of an analog of MCP-3, RANTES or MIP-1 α for a time and
10 under conditions sufficient to inhibit the biological activity of the native molecules.

Another aspect of the present invention is directed to a method of treating inflammatory diseases in a mammal suffering from said diseases comprising
15 administering to said mammal a therapeutically effective amount of the analog of the present invention.

A further aspect of the present invention is directed to a method of treating autoimmune disorders
20 in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an analog of the present invention.

Another aspect of the present invention is directed to pharmaceutical compositions comprising an
25 antagonistically effective amount of one or more of the aforementioned analogs and a pharmaceutically acceptable carrier.

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BRIEF DESCRIPTION OF THE FIGURES

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Figure 1A is a graph showing NH₂-terminal truncation of RANTES and MCP-3 resulted in a loss of chemotactic activity on THP-1 cells.

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Figure 1B is a graph showing the NH₂-terminal truncated RANTES analogs (RANTES(6-68), RANTES(7-68), RANTES(8-68), RANTES(9-68), and RANTES(10-68)) had no detectable activity.

10

Figure 1C is a graph showing truncation of the residues 1-9 of MIP-1 α (MIP-1 α (10-70)) reduced activity by more than 95% of the native MIP-1 α , further truncation resulted MIP-1 α (11-70) which

15 completely lost chemoattractant activity on THP-1 cells.

Figure 2A is a graph showing RANTES antagonist activity of the RANTES(9-68) analog titrated at the indicated concentrations against RANTES and MCP-3 in a chemotaxis assay using THP-1 cells.

Figure 2B is a graph showing MCP-3 antagonist activity of the MCP-3(10-76) analog titrated at the indicated concentrations against MCP-3 and RANTES in a chemotaxis assay using THP-1 cells.

Figure 2C is a graph showing the inhibition of MIP-1 α induced (10 nM or 10⁻⁸M) THP-1 migration by MIP-1 α (10-70) and MIP-1 α (11-70).

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Figure 2D is a graph showing the inhibition of RANTES-
1 induced (10 nM or 10^{-8} M) THP-1 migration by MIP-1 α (10-
70) and MIP-1 α (11-70).

Figure 3A is a graph showing RANTES antagonist
5 activity of RANTES(9-68) titrated at the indicated
concentrations against RANTES-induced and MCP-3-
induced N-acetyl- β -D-glucosaminidase release using
human blood monocytes.

10 Figure 3B is a graph showing MCP-3 antagonist activity
of MCP-3(10-76) titrated at the indicated
concentrations against MCP-3-induced and RANTES-
induced N-acetyl- β -D-glucosaminidase release using
human blood monocytes.

15 Figures 3C and 3D are graphs showing MIP-1 α antagonist
activity of MIP-1 α (10-70) and MIP-1 α (11-70) titrated
at the indicated concentrations against MIP-1 α -induced
and RANTES-induced-N-acetyl- β -D-glucosaminidase
20 release using human blood monocytes. The
concentration of MIP-1 α and RANTES used was 30 nM.

Figures 4A and 4B are graphs showing competitive
binding to THP-1 cells of unlabeled RANTES and
25 RANTES(9-68) titrated at the indicated concentrations
in the presence of 4 nM labeled RANTES (A), and 4 nM
labeled MCP-3 (B).

Figures 4C and 4D are graphs showing competitive
30 binding to THP-1 cells of unlabeled MCP-3 and MCP-

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3(10-76) titrated at the indicated concentrations in
1 the presence of 4 nM labeled MCP-3 (D) and 4 nM
labeled RANTES (C).

Figure 4E is a graph showing competitive binding to
5 THP-1 cells of unlabeled MCP-3 and MCP-3(8-76), MCP-
3(9-76), MCP-3(10-76) and MCP-3(11-76) titrated at the
indicated concentrations in the presence of 4 nM
labeled MCP-3.

10 Figure 4F is a graph showing competitive binding to
THP-1 cells of unlabeled RANTES and RANTES (6-68),
RANTES (7-68), RANTES (8-68), RANTES (9-68) and RANTES
(10-68) titrated at the indicated concentrations in
the presence of 4 nM labeled RANTES.

15 Figure 5 is a graph showing competitive binding to
THP-1 cells of unlabeled MIP-1 α and MIP-1 α (10-70) and
MIP-1 α (11-70) titrated at the indicated concentrations
in the presence of 4 nM labeled MIP-1 α .

20 Figure 6 is a chart summarizing the binding and
antagonist activities of MIP-1 α , RANTES and MCP-3
analogs.

25 Figure 7 is a graph showing competitive binding to
THP-1 cells of unlabeled RANTES and RANTES(9-68) and
RANTES(9-68, P9T) titrated at the indicated
concentrations in the presence of 4 nM labeled MCP-1.

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Figure 8 is a graph which shows that human RANTES(9-
1 68) was able to bind to the receptor on mouse
peritoneal macrophages. Native human RANTES did not
significantly bind to mouse peritoneal macrophages.

5 Figures 9A and 9B are graphs showing that daily i.p.
injections of RANTES (9-68) inhibited the swelling and
the incidence of arthritis in treated mice.

Figure 10 is a chart showing untreated control animals
10 and animals administered a peptide control developed
rheumatoid arthritis, substantial infiltration of
mononuclear cells into the synovial tissue, extensive
hyperplasia of the synovial lining, pannus formation
and bone and cartilage damage. In contrast, animals
15 treated with RANTES(9-68) exhibited no invasive
pannus, no bone or cartilage damage and insignificant
inflammatory infiltration.

Figure 11 is a graph which shows that human MCP-3 (9-
20 76) was able to bind to the receptor on mouse
monocytes. Native human MCP-3 did not significantly
bind to mouse monocytic cells.

Figure 12 is a graph showing that daily i.p.
25 injections of MCP-3 (9-76) inhibited the swelling and
the incidence of arthritis in treated mice.

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DETAILED DESCRIPTION OF THE INVENTION

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One aspect of the present invention is directed to NH₂-terminally truncated analogs of three human chemokines, MCP-3, RANTES and MIP-1 α , useful for
5 treating inflammatory conditions and autoimmune disorders. Inflammatory conditions contemplated by the present invention include both acute and chronic inflammatory diseases. Examples include, but are not limited to, arthritis, asthma, colitis, psoriasis,
10 atheromas and the like. Examples of autoimmune conditions include rheumatoid arthritis and multiple sclerosis and the like. As defined by the present invention an NH₂-terminally truncated chemokine analog acts as an antagonist to a corresponding native
15 chemokine. The antagonistic activity of the chemokine analogs of the present invention includes inhibition of biological activity induced by corresponding native chemokines.

In the application, the products of the
20 present invention are referred to by various terms, including "analogs of the present invention", "NH₂-terminally truncated analogs", "polypeptides of the present invention", "antagonists of the corresponding chemokines", etc. are used interchangeably and denote
25 equivalent expression.

As defined by the present invention biological activity refers to the biological activity of the native chemokines, as measured by standard assays, including but not limited to receptor binding,
30 chemotaxis, calcium mobilization and exocytosis

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characterized by N-Acetyl- β -D-glucosaminidase release
1 and elastase release.

Preferred MCP-3 analogs of the present
invention include: MCP-3 (7-76), (8-76), (9-76), (10-
76) and (11-76). Preferred RANTES analogs of the
5 present invention include: RANTES (6-68), (7-68), (8-
68), (9-68) and (10-68). Preferred MIP-1 α analogs of
the present invention include: MIP-1 α (9-70), (10-70)
and (11-70).

As defined by the present invention any
10 reference by number to an amino acid in an analog,
e.g., MCP-3 analog will be a reference to the
corresponding residue number from the amino acid
sequence of the native molecule shown in Table 1. For
example, where the first 7, 8, 9 or 10 amino acids of
15 MCP-3 are truncated (as is the case for the MCP-3
analogs shown in Table 1) the analogs will be referred
to MCP-3 (8-76), MCP-3 (9-76), MCP-3(10-76) and MCP-
3(11-76), respectively.

The analogs of the present invention
20 comprise an amino acid sequence that is identical to
the corresponding region of the native molecule, or a
polypeptide fragment having a region that is
substantially homologous to all or part of a region of
the corresponding native molecule while still
25 maintaining the antagonist activity to the
corresponding native molecule. The analogs of the
present invention maintain the ability to
competitively bind at the receptor site of the
corresponding chemokine. Analogs which are
30 "substantially homologous" to all or part of a region

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of the native molecule are characterized as having
1 from 1-10 amino acid deletions, additions or
substitutions that do not result in an analog losing
its ability to compete with a native chemokine for
binding to a chemokine receptor while still retaining
5 the antagonist activity. It is preferred that the
MCP-3 analogs of the present invention are at least
about 80% homologous to native MCP-3 and can have as
much as 100% homology to native MCP-3, inclusive.
More preferably, MCP-3 analogs of the present
10 invention are at least about 85% homologous to native
MCP-3. Still more preferably, MCP-3 analogs of the
present invention are at least about 90% homologous to
native MCP-3. Most preferred MCP-3 analogs of the
present invention are at least about 95% homologous to
15 native MCP-3. RANTES and MIP-1 α analogs of the
present invention are preferably at least about 60%
homologous to the corresponding native polypeptides
and can extend to as much as 100% homology to the
corresponding native polypeptides, inclusive. The
20 RANTES analogs and MIP-1 α analogs of the present
invention are preferably at least about 60% homologous
to the native polypeptides and more preferably at
least about 75%, even more preferably at least about
85% and most preferably at least about 90% and 95%
25 homologous to the native chemokine.

Polypeptides having truncation at both the
N-terminal end and the C-terminal end are also
contemplated to be within the scope of the present
invention. For example, up to 10 amino acids can be

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truncated from the C-terminal end of the analogs
1 described hereinabove.

It is noted that it is well known in the art
that certain amino acids can be replaced with others
resulting in no or relatively little change in the
5 properties of the polypeptide. Thus, for example,
specific amino acid substitutions that may be
tolerated in each analog of the present invention
include: Glu or Asn for Asp; Asp or Gln for Glu; Arg
for Lys; Lys for Arg; Asn for His; Pro for Gly; Gly or
10 Thr for Pro; Asn or Met or Leu for Gln; Gln, Ser or
Ala for Asn; Ser, Val or Ile or Pro for Thr; Thr or
Ala for Ser; Phe for Tyr; Tyr for Phe; Ile, Val, or
Met for Leu; Ser for Ala; and any combinations
thereof. As defined by the present invention, e.g.
15 "P9T" denotes a substitution of the amino acid Proline
for the amino acid Threonine at position 9 of the
native molecule. Similar modifications denoted by the
single amino acid code followed by a molecule position
number and the substituting amino acid are
20 contemplated by the present invention (see Figure 9).

The present inventors have found that there
are certain portions of the polypeptides of the
present invention that must be maintained and cannot
be modified. More specifically, deletion of certain
25 amino acids from the native chemokines and the
antagonists of the present invention will result in a
loss of receptor binding capability. For example,
deleting the disulfide bridge in MCP-3 or MCP-3
analogs (i.e. the cysteines at positions 12 and 52)
30 will result in conformational changes and will

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preclude receptor binding. Similar results have been
1 discovered in RANTES (i.e. deletion of cysteine 11 and
50) and MIP-1 α (i.e. deletion of cysteine 12 and 51).

The inventors have also discovered that
extensive substitutions may be made to the C-terminal
5 region of e.g. MCP-3 of residues 23-76 without
significant loss of MCP-3 receptor binding. However,
it is preferred that amino acids 13-22 of an MCP-3
analog are the same as in the native MCP-3.

The analogs are synthesized using art
10 recognized techniques in peptide chemistry. For
example, they may be synthesized by adding one amino
acid at a time to an amino acid or peptide. The amino
acids or peptide contain the appropriate protecting
groups on the side chains and on the N-terminal
15 portions thereof. The first amino acid containing the
appropriate protecting group on the N-terminus and the
side chain is coupled to a second amino acid having a
protecting group on the side chain and the C-terminal
end in the presence of a peptide coupling reagent,
20 such as DCC to form the resulting peptide.
Preferably, the C-terminal end is bound to a resin so
that the peptide is built from the C-terminal end to
the N-terminal end thereof. After the peptide is
formed, another N α -protected amino acid having side
25 chain protection is coupled to the peptide formed
previously. This process is continued until the
desired peptide is formed; then the protecting groups
are removed by art recognized techniques and the
peptide is removed from the resin by art-recognized
30 techniques.

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An exemplary procedure for polypeptide
1 synthesis of the compounds of the present invention is
as follows. The analogs are preferably synthesized
using tBoc chemistry on a peptide synthesizer and
purified using reverse phase HPLC. The synthesis is
5 started with a protected C-terminal amino acid linked
to a cross-linked polystyrene resin via a 4-
(carboxamidomethyl) benzyl ester linkage (the so-
called pam resin) (0.4 mmol of 0.8 mmol/g of aminoacyl
resin). N α -t-Boc acids with appropriate side chain
10 protecting groups are added in a stepwise fashion
until the entire protected polypeptide chain is
formed. The groups utilized for side chain protection
are those commonly used in this art. Examples
include: benzyl (Asp, Gly, Ser and Thr); 4-
15 methylbenzyl (Cys); toluenesulfonyl (Arg); 2-
chlorobenzoyloxycarbonyl (Lys); 2-
bromobenzoyloxycarbonyl (Tyr); formyl (Trp);
benzyloxymethyl (His); and none (Ala, Asn, Gly, Gln,
Ile, Leu, Met, Phe, Pro, Val). Samples may be taken
20 after each step to retrospectively monitor the amino
acid coupling yields using a ninhydrin-based reaction
following the procedures of Sarin, et al. (1981) Anal.
Biochem. 117:147-157. The resin is dried and cleaved
using the "low-high" hydrogen fluoride method as
25 described by Tam, et al. (1984) J. Am. Chem. Soc.
105:6442-6485 (the contents of which are incorporated
herein by reference), except for the following
modifications: after the 25% hydrogen fluoride step,
the partially protected peptide resin is filtered from
30 the reaction mixture by using an all-Teflon filtration

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apparatus fitted with a Zitex filter and washed with
1 dichloromethane and dried before the high 90% hydrogen
fluoride step. The ethyl acetate precipitate of the
material released from the resin is dissolved in 50 ml
of 6 M guanidine hydrochloride, 0.1 M Tris-acetate, pH
5 8.5, and 10% 2-mercaptoethanol. This mixture is the
crude peptide product.

Alternately, histidine may be protected with
p-benzyloxymethyl instead of dinitrophenyl. The p-
benzyloxymethyl group is acid labile, thus eliminating
10 the need for thiolysis of the dinitrophenyl group
before and after hydrogen fluoride deprotection.
Acetylation is carried out on the N α deprotected, but
otherwise fully protected peptide resin, using acetic
anhydride (50%) in dimethylformamide.

15 The crude peptide product may be purified
and folded by art recognized techniques. The
following protocol is exemplary: three different C-18
silica HPLC columns may be used in the purification
and analysis of the peptide, including a preparative
20 column (22.4 x 250 mm column with a 22.4 x 100 mm
guard column) packing with 12 μ m, 300-A pore size
packing (Dynamax, Rainin Instrument Co., Woburn, MA);
a semipreparative column (10 x 250 mm) Vydac C018
column, with 5- μ m particle, 300-A pore-size packing
25 (Separations Group, Hesperia, CA); and an analytical
column (4.6 x 250 mm) (Vydac) containing the same
packing. The crude peptide product is first acidified
to pH 4.0 with 20% acetic acid and filtered. The
crude peptide product is then loaded onto the
30 preparative column and the retained material eluted

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with a 0-60% water-acetonitrile gradient in 0.1%
1 trifluoroacetic acid over 4 hours at a flow rate of 15
ml/min. A sample (25 µl) of fractions containing 225-
nm UV-absorbing material is rerun on the analytical
column and by comparison with the profile of the crude
5 material, fractions containing the major peak are
pooled and lyophilized.

To fold the protein, the material is
reconstituted in 1 M guanidine hydrochloride and Tris-
acetate, pH 8.5, in 10% dimethyl sulfoxide (DMSO) at a
10 concentration of 0.2 mg/ml and stirred overnight in a
covered beaker. MIP-1α is folded as above in the
absence of DMSO. MIP-1α (10-70) and MIP-1α (11-70)
are folded in 10% DMSO in water. This procedure
promotes formation of the disulfide bridges by
15 oxidation of the appropriate half-cysteines. The
materials are acidified with 2 ml of acetic acid, and
half is loaded onto the semipreparative column and the
retained material is eluted with the same gradient as
described above at a flow rate of 3 ml/min. Samples
20 of each fraction are run on the analytical column.
Fractions containing only material with the retention
time of the major peak in the folded material are
pooled and lyophilized.

An assay for free sulfhydryls using Ellman
25 reagents, as described by Clark-Lewis, et al. (1988)
Proc. Natl. Acad. Sci. USA 65:7897-7902, may be used
to determine the extent of folding. In addition,
folding may be monitored on the analytical HPLC column
by observing the appearance of a peak corresponding to

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the folded form that has a retention time

1 approximately 3 minutes earlier than the reduced form.

 Analog purity may be assessed on an analytical HPLC column or by other means such as isoelectric focusing. An exemplary protocol for
5 isoelectric focusing is as follows. Mini polyacrylamide gels (Pharmacia PHAST gels, IEF 3-9; Pharmacia, Uppsala, Sweden) are washed in 8 M urea and then in 8 M urea containing pH 9-11 Ampholytes (Pharmacia), for 30 minutes each, either with or
10 without 10 μ M dithiothreitol. Gels are prerun for 15 V-h at 200-V, 2.0-mA, 3.0-mW maximum settings, and the samples are loaded and run for 410 V-h at 1000-V, 5.0-mA, 3.0-mW maximum settings on the Pharmacia PHAST systems for a total of 500-V with maximum settings of
15 2.0-mW, 5.0-mA and 1000-V. The pH gradient may be determined by using a surface pH electrode. The gels may be stained with silver by using the PHAST developing systems as described in the Pharmacia manual.

20 Sequencing of analogs may be determined by protein sequencing, for example by using the following protocol. Protein sequences are determined by Edman degradations using either solid-phase or gas-liquid-phase methods. For solid-phase sequence analysis,
25 reduced and carboxymethylated protein or proteolytic cleavage fragments are coupled to arylamine-functionalized poly(vinylidenedifluoride) membranes (Sequelon AA; Milligen/Bioscience, Burlington, MA) using the water-soluble carbodiimide 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride and
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sequenced in a Milligen/Biosearch Model 6600 sequencer
1 using standard protocols. For gas-liquid-phase
sequence analysis, polypeptides may be applied to
Polybrene-coated glass fibre disks and sequenced in an
Applied Biosystems Model 477 protein sequencer using
5 standard protocols. Sequencing of protected peptide
resins may be carried out on N α -deprotected samples by
using the same methods. N-terminal solid-phase
sequencing runs usually reveal a major portion of the
sequence. The remaining sequence is obtained by runs
10 of the HPLC-fractionated fragments, derived either by
proteolytic cleavage with Asp-N-endoprotease
(Boehringer Mannheim Canada, Laval, Quebec) or by
chemical cleavage, through preferential hydrolysis of
the Asp-Pro peptide bond in dilute formic acid.

15 Molecular weight of the synthetic proteins
prepared as described above are determined by art-
recognized techniques, such as electrospray mass
spectrometry on a SCIEX triple quadrupole Mass
Spectrometer equipped with a liquid delivery
20 apparatus. The molecular mass from the peaks
corresponding to the charge to mass ratios of the
different multiple ionized species of the protein can
also be analyzed as described by Convey, et al. (1988)
Rapid Commun. Mass. Spectrom. 2:249-256.

25 The chemokine analogs of this invention may
also be prepared through recombinant means, with
expression in mammalian or non-mammalian systems.
Portions of a DNA sequence encoding, e.g., MCP-3, are
appropriately modified to produce the desired analog
30 when the DNA sequence is expressed. Methods and

-20-

protocols for preparation and expression of such
1 recombinant DNA are known in the art, including the
protocol described by Masure, et al. (1995) J. Interferon Cytokine Res. 15:955-963 used for
production of mutant MCP-3 proteins, incorporated
5 herein by reference.

In accordance with the present invention,
the inventors have discovered that, in contrast to the
full-length native forms, the analogs of the present
invention were antagonists to the corresponding native
10 molecules. Moreover, they lacked detectable
chemoattractant activity for the receptor carrying
cells. For example, RANTES (9-68), MCP-3 (10-76) and
MIP-1 α (10-70) lacked detectable chemoattractant
activity for human THP-1 monocytic cells. RANTES (6-
15 68), (7-68), (8-68) and (10-68) also lacked
chemoattractant activity for T-cells, monocytes, NK
cells, basophils and eosinophils. MCP-3 (8-76), (9-76)
and (11-76) also lacked activity for human CD4+ and
CD8+ T lymphocytes, NK cells, eosinophils and
20 basophils. MIP-1 α similarly lacked chemoattractant
activity for monocytes and human T-cells.

In accordance with the present invention,
the inventors have observed certain regions within the
native chemokines which are essential for their
25 functional activities. Specifically, NH₂-terminal
amino acid residues within the 1-5 region of native
RANTES, residues within the 1-7 region of native MCP-3
and residues within the 1-9 region of native MIP-1 α
are critical for chemoattractant activity and receptor
30 activation, for example. However, by truncating the

1-5 region of native RANTES, 1-7 region of native MCP-
1 3 and 1-9 region of native MIP-1 α , the resulting
molecules become antagonists to the native chemokines.

The inventors have also discovered that the
truncated chemokine analogs of the present invention
5 act as antagonists to native, full-length chemokines
and do not possess the chemoattractant activity. For
example, RANTES(9-68), MCP-3(10-76) and MIP-1 α (10-70)
inhibited the biological activity (e.g.
chemoattractant activity and N-acetyl- β -D-
10 glucosaminidase release, elastase release and
intracellular calcium mobilization) induced by the
corresponding native forms.

In accordance with the present invention,
the inventors have assessed the receptor-binding
15 specificity of the truncated analogs. The inventors
have discovered significant cross-receptor binding
among the identified chemokine analogs of the present
invention. Thus, for example, unexpectedly, RANTES
(9-68) inhibited native RANTES, MCP-3, MCP-1 and MIP-
20 1 α -induced chemotaxis and N-acetyl- β -D-glucosaminidase
release and intracellular calcium mobilization.

The inventors also observed that MCP-3 (9-
76) and MCP-3 (10-76) inhibited native MCP-3-induced,
RANTES-induced, MCP-1-induced, calcium mobilization,
25 monocyte N-acetyl- β -D-glucosaminidase release and
chemoattractant activity.

The inventors have further observed that
MIP-1 α (10-70) inhibited native MIP-1 α / β -induced and
RANTES-induced monocyte N-acetyl- β -D-glucosaminidase
30

release and chemoattractant activity and calcium
1 mobilization.

The present inventors have determined that
the polypeptides (analogs) of the present invention
act as antagonists to the effects of native RANTES,
5 MIP-1 α and MCP-3 in various tests, such as chemotaxis,
calcium mobilization, receptor binding in THP-1 cells
(a monocytic cell line). Based upon these
observations, the inventors concluded that the
polypeptides of the present invention are useful in
10 blocking the effects of native RANTES, MIP-1 α and MCP-
3 molecules and thus are useful as therapeutic agents.
A preferred use of the polypeptides of the present
invention is in blocking the effects of RANTES, MIP-1 α
and MCP-3 in the recruitment and/or activation of pro-
15 inflammatory cells. Thus, the present invention has
utility in the treatment of such inflammatory diseases
as asthma, allergic rhinitis, colitis, psoriasis,
atheromas, dermatitis and the like. In addition, the
polypeptides are also useful in the treatment of
20 autoimmune diseases, e.g., rheumatoid arthritis. For
example, the inventors have observed that the analogs
of the present invention (e.g. RANTES antagonists) are
useful in treating autoimmune disorders (e.g.,
rheumatoid arthritis) in mammals. The inventors have
25 determined that the analogs of the present invention
prevent the onset of rheumatoid arthritis in MRL-1pr
mice. The inventors have discovered that
administration of e.g., RANTES(9-68) significantly
inhibited the clinical incidence of adjuvant enhanced
30 arthritis for 30 days in an autoimmune mouse model

(MRL-1pr mice have lupus erythematosus and rheumatoid
1 arthritis similar to the corresponding human
diseases).

In another aspect of the present invention,
methods for inhibiting the biological activity of
5 native chemokines are provided which involve
administering a therapeutically effective amount of a
truncated analog of the present invention to a mammal
to treat, e.g. inflammatory diseases and autoimmune
diseases.

10 In another aspect of the present invention,
the NH₂-terminally truncated chemokine analogs of the
present invention are administered in pharmaceutical
compositions. The analogs of this invention may be
administered as a nasal spray for upper respiratory
15 treatments or as an aerosol inhaler for lung
conditions. The analogs may also be used in topical
applications. The analogs may also be administered
via an osmotic pump or in a sustained release
formulation. Alternatively, the analogs of the
20 present invention may be delivered by injection. It
is preferred that the injection is intramuscular or
intraanal. However, the present invention also
contemplates intradermal, intraperitoneal or
intraarticular injections as well as long term
25 continuous delivery via subcutaneous osmotic pump or
sustained release formulations.

Other methods of in vivo and in vitro use of
the chemokine analogs of the present invention will be
readily apparent from the examples herein and the
30 assays described above. The chemokine analogs of the

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present invention are present in the various
1 pharmaceutical formulations described hereinabove in
an "antagonistically effective amount" or
"therapeutically effective" amount. "Antagonistically
effective amount" or "therapeutically effective"
5 amount as used herein is defined as an amount of
chemokine analog sufficient to significantly inhibit
the biological activity of native chemokines but low
enough to avoid serious side effects such as toxicity,
for example (at a reasonable risk/benefit ratio)
10 within the sound medical/scientific judgment of the
skilled artisan. However, it is preferred that the
dose of the formulation contains between 0.01-1 mg/kg
for a nasal spray and 0.1-10 mg/kg for other forms of
delivery. It is most preferred that the formulation
15 contains between 0.01-0.1 mg/kg for a nasal spray and
0.1-1 mg/kg for other forms of delivery. The dosage
schedule of the chemokine analogs will typically be
determined (at a reasonable risk/benefit ratio) within
the sound medical/scientific judgement of the skilled
20 artisan. However, it is preferred that the chemokine
analogs are administered at least about 3 times per
week. It is most preferred that the chemokine analogs
are administered on a daily basis.

The exact dose of a chemokine analog of the
25 present invention to be used in a particular
application may be determined by accepted
pharmaceutical methods known to the skilled artisan.
This is accomplished by conventionally measuring the
concentration of analog in the blood and determining
30 the analog half-life. Without wishing to be bound, it

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is believed that the analogs of the present invention
1 have what would otherwise be an unexpectedly high half
life for similar polypeptides due to molecular
stability at body temperature and due to the high
binding affinity to their receptors.

5 Pharmaceutical compositions comprising the
analogs of the present invention additionally contain
pharmaceutical vehicles, such as carriers and
adjuvants described in the literature of
pharmaceuticals and related fields. The analogs of
10 the present invention are formulated by the skilled
artisan, using art-recognized techniques, taking into
consideration the nature of the polypeptide compounds
and the desired mode of administration. The product
of the present invention are soluble and are therefore
15 readily formulated in physiological buffers, e.g.
physiological saline.

The inventors have observed that unlike
other chemokines, native human RANTES and MIP-1 α form
aggregates in physiological conditions (e.g. at about
20 pH 7.2) at concentrations as low as 100 nM. This
presents a significant hurdle to in vivo applications
because therapeutic efficacy usually requires much
higher concentrations of the active compounds (i.e.,
up to 2 mM). However, the present inventors have
25 surprisingly discovered that the truncated chemokine
analogs of the present invention exhibit no
aggregation at concentrations above about 1 μ M.
Therefore, the truncated chemokine analogs (e.g.
RANTES and MIP-1 α) of the present invention are highly

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soluble, and thus facilitate more in vivo applications
1 than their native counterparts.

In addition, the chemokine analogs of the
present invention can also formulated in sustained
release delivery systems or topical formulations
5 containing an aqueous component.

MCP-3, RANTES and MIP-1 α analogs of the
present invention are assayed for biological activity
by use of a cytosolic-free calcium assay, a chemotaxis
assay using cells of monocytic origin, or by other
10 conventional assays for MCP-3, RANTES or MIP-1 α
activity including but not limited to: assays to
determine exocytosis of leukocytes such as elastase
release and N-acetyl- β -D-glucosaminidase release,
superoxide production, histamine release, LTC₄ release
15 and the like.

The invention will now be illustrated by
means of the following non-limiting examples.

In the following example, analysis of
cytosolic-free calcium was carried out using the
20 following protocol. Cells (4×10^5) are loaded with
12, 5 μ g/ml Fluo-3AM or Fluo-2 in PBS saline with 0.38
mg/ml Pluronic F127 (Molecular Probes, Eugene, OR) at
37°C for 30 minutes. After washing with PBS, the
cells were resuspended in 25 mM Hepes, 140 mM NaCl, 10
25 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, and 3 mM KCl, pH
7.3. The fluorescence was monitored at 7 second
intervals over 150 seconds, after addition of test
sample. Maximum Ca²⁺ levels were established using
Fluo-3AM or Fluo-2 (Designated 100% saturation) for
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each set of measurements by addition of 5 μ M Ionomycin
1 (Sigma Chemical Co., St. Louis, MO).

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EXAMPLE 1

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Representative polypeptides of the present
invention were tested for their functional activity
according to the procedure described hereinabove. The
5 results are as follows. In contrast to the full-
length native forms, RANTES (9-68), MCP-3 (10-76) and
MIP-1 α (10-70) lacked detectable chemoattractant
activity for human THP-1 monocytic cells (Figures 1A-
1C). In addition, for human blood-derived monocytes,
10 neither chemotaxis nor N-acetyl- β -D-glucosaminidase
release was detected up to 1 μ M. RANTES (6-68), (7-
68), (8-68) and (10-68) as well as MCP-3 (8-76), (9-
76) and (11-76) also lacked chemoattractant activity
(Figure 1B). MIP-1 α (10-70) had only minor activity
15 (about 3% of that of the corresponding native MIP-1 α).
MIP-1 α (11-70) had no detectable activity. Thus,
residues within the 1-5 region of native RANTES, 1-7
region of native MCP-3 and 1-9 region of native MIP-1 α
are essential for the functional activities of the
20 native chemokines. Truncation of these regions
resulted in a complete loss of e.g. chemoattractant
activity of the native chemokines.

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EXAMPLE 2

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Inhibition of native MCP-3, RANTES or MIP-1 α mediated chemotaxis are determined by using the aforementioned chemotaxis assay. Constant amounts of

5 MCP-3, RANTES or MIP-1 α (e.g. 10 nM) are added to each well, and the analogs are titrated in the assay.

Cell preparations for use in the aforementioned assays consist of human monocytes, or monocytic cell lines such as the cell line THP-1.

10 THP-1 were obtained from American Type Culture Collection (Rockville, MD) and may be, optionally, maintained in RPMI 1640 medium supplemented with 10% FCS.

Human monocytes were isolated from buffy

15 coats of normal donor blood by the following protocol. A cell suspension was loaded onto Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifugated at 400 g for 25 minutes followed by density centrifugation on a discontinuous Percoll (Pharmacia) gradient at 500 g

20 for 30 minutes. Cell fractions with a density of 1.051-1.053 (g/ml) were generally greater than 70% monocytes by morphology and were used in the assay.

RANTES (9-68), MCP-3 (10-76), MIP-1 α (10-70) and MIP-1 α (11-70) were tested for their antagonist

25 activities. All three inhibited the activity induced by the corresponding native forms (Figures 2A-2D). RANTES (9-68) fully blocked the chemoattractant activity of RANTES for THP-1 cells, and 41 nM was required to inhibit the agonist activity of 10 nM

30 native RANTES by 50% (IC₅₀ = 41 nM). Similar results

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were obtained for migration of peripheral blood
1 monocytes. The ability of RANTES (9-68) to inhibit
chemokine-induced exocytosis from human blood
monocytes was assessed by measuring the release of N-
acetyl- β -D-glucosaminidase. A 10-fold lower
5 concentration (IC_{50} = 4 nM) of RANTES (9-68) was
required to inhibit RANTES-induced enzyme release than
was needed for similar inhibition of chemotaxis
(Figure 3A).

To test the specificity of the three
10 truncated antagonists, their ability to inhibit the
activities induced by RANTES, MCP-3 and MIP-1 α was
examined (Figures 3A-3D). The RANTES (9-68) analog
completely inhibited both MCP-3 and MIP-1 α induced
chemotaxis and N-acetyl- β -D-glucosaminidase release.
15 The respective IC_{50} values were 200 nM and 126 nM for
THP-1 cell chemotaxis and 170 nM and 20 nM for
monocyte release activity (Figure 6). Thus RANTES (9-
68) inhibited all the chemokines, but it had the
highest potency for RANTES.

20 MCP-3 (10-76) was found to inhibit MCP-3
induced monocyte N-acetyl- β -D-glucosaminidase release
with an IC_{50} of 37 nM and also chemoattractant activity
 IC_{50} 470 nM. Thus it was less potent than RANTES (9-
68) for RANTES-stimulated activities. However, MCP-3
25 (10-76) also inhibited RANTES-induced and MIP-1 α -
induced activities with similar effectiveness to its
inhibition of MCP-3 elicited function (Figure 3B).
Furthermore, MCP-3 (10-76) blocked enzyme release from
monocytes. The IC_{50} values for inhibiting both RANTES
30 or MCP-3 induced release were around 10-fold lower

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than for chemotaxis of either THP-1 cells or
1 monocytes. Another MCP-3 variant, MCP-3 (9-76), was
2-3 fold more potent than MCP-3 (10-76) in all the
inhibition assays.

MIP-1 α (10-70) was found to inhibit MIP-1 α
5 or RANTES induced monocyte-N-acetyl- β -D-
glucosaminidase release with an IC₅₀ of 1 nM (Figures
3C-3D). MIP-1 α (10-70) was also found to inhibit
monocyte/THP-1 cell migration. MIP-1 α (11-70) also
inhibited both chemoattractant activity and enzyme
10 release of native MIP-1 α and native RANTES with an IC₅₀
of 30 nM and 10 nM, respectively. Because MIP-1 α
antagonists did not inhibit MCP-3 activity, MIP-1 α
antagonists are relatively selective for MIP-1 α and
RANTES. Antagonists of native RANTES and native MCP-3
15 have relatively broad inhibitory spectra.

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EXAMPLE 3

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MCP-3, RANTES or MIP-1 α receptor binding are determined by the following protocol. MCP-3 (10 μ g) were labeled with monoiodinated Bolton-Hunter reagent (specific activity 2,200 Ci/mmol; DuPont, Wilmington, DB) at 4°C for 20 minutes, to provide specific activity of, e.g. 125 I-labeled MCP-3 of 150 Ci/mmol. RANTES and MIP-1 α were labeled using lactoperoxidase. One mCi (3.7 MBq) of Na 125 I (ICN Biomedicals, Irvine CA) and 1 μ g of lactoperoxidase (80-150 units-mg $^{-1}$, Sigma) were added to 5 μ g of RANTES or MIP-1 α in 50 μ l of 0.5 M sodium acetate, pH 6.5 at room temperature for 3 min. Saturated tyrosine (150 μ l) was added to stop the reaction, and the proteins were separated from the label by Sephadex G-25 chromatography. The specific activity of 125 I-labeled RANTES was 260 Ci-mmol $^{-1}$.

To determine the binding kinetics, monocytic cells (such as THP-1) at (5×10^6 cells) in 200 μ l of binding buffer (RPMI 1640, 0.5 mg/ml BSA, 50 mM Hepes and 0.01% NaI) were incubated with varying concentrations of 125 I-MCP-3 at 4°C for 30 minutes. The cells were pelleted through a mixture of diacetylphthalate and dibutylphthalate and radioactivity that is cell associated was counted (total binding). Nonspecific binding was determined in the presence of a 100-fold concentration of unlabeled ligand and subtracted from the total binding. Kinetic parameters (K_d and receptor number) were determined by Scatchard analysis.

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Competitive receptor binding by, e.g. MCP-3,
1 analogs is measured by carrying out the aforementioned
receptor binding protocol wherein various
concentrations of e.g. MCP-3 analogs are added to the
cells in the presence of e.g., 4 nM ^{125}I -MCP-3. Non-
5 specific binding is subtracted from total binding and
the result is expressed as a percent of maximum
specific binding (See Figures 4A-4E and Figure 5).

Competition binding studies were performed
to determine if the observed antagonist properties
10 correlated with their interaction with chemokine
binding sites.

The RANTES (9-68) analog competed for
binding of labeled RANTES. From this data, the
dissociation constant (K_d) was calculated to be 19 nM,
15 that is only about 4-5 fold of that of native RANTES
binding affinity (4 nM). Furthermore, RANTES (9-68)
also displaced labeled MCP-3 ($K_d = 57$ nM). Although,
as expected, native RANTES competed strongly for
RANTES binding, its competition for labeled MCP-3 was
20 very weak and insufficient to derive a K_d value.
Thus, truncation of RANTES resulted in a markedly
increased affinity for MCP-3 binding sites.

MCP-3 (10-76) competed for MCP-3 binding
with a K_d of 57 nM and also competed for binding of
25 both RANTES ($K_d = 50$ nM) and MIP-1 α . The competition
of the MCP-3 (10-76) analog for labeled MCP-3 was only
2-fold weaker than that of full-length MCP-3. For
RANTES receptors, MCP-3 and MCP-3 (10-76) had about
the same affinity. The results indicate that MCP-3
30 (10-76) had similar affinity for the binding sites of

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all three chemokines. Similar receptor binding
1 affinity data were obtained with MCP-3 (9-76). MCP-3
(8-76); MCP-3 (7-76) and MCP-3 (6-76) had lower
receptor binding affinities than MCP-3 (9-76) and MCP-
3 (10-76).

5 The MIP-1 α (10-70) analog competed for
binding of labeled MIP-1 α with a K_d of 25 nM and also
competed for binding of RANTES (K_d = 14 nM) and less
for MCP-3 (K_d = 281 nM). Native MIP-1 α and MCP-1 α
(10-70) had about the same affinity for RANTES
10 receptors. MIP-1 α (11-70) competed only for RANTES
and MIP-1 α receptor binding. No competition for MCP-3
was observed. Therefore MIP-1 α (10-70) has higher
inhibitory potency than MIP-1 α (11-70).

A further assay that may be carried out to
15 determine whether a non-chemotactic analog binds to,
e.g. MCP-3 receptors is to measure the ability of an
analog to desensitize calcium mobilization by MCP-3.
Following a first treatment with a MCP-3 receptor
ligand, the calcium response is temporally
20 desensitized to a second treatment with a MCP-3
receptor ligand. This is determined by carrying out
the aforementioned cytosolic-free calcium assay with
addition of a first ligand, followed by a second
treatment after 60 seconds using either the same of a
25 different ligand. A MCP-3 antagonist will not of
itself stimulate calcium induction but when used as
the first ligand, will desensitize the cells to
subsequent stimulation by MCP-3. The following
example illustrates the result.

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EXAMPLE 4

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The truncated analogs RANTES (9-68) and MCP-3 (10-76) were tested for their ability to inhibit the transient rise of $[Ca^{2+}]$ induced in monocytes by native RANTES and MCP-3. In all cases the truncated analogs inhibited the $[Ca^{2+}]$ rise induced by the native chemokines. The most potent effect was observed with RANTES (9-68), which at 30 nM totally prevented the $[Ca^{2+}]$ changes induced by 10 nM RANTES. For inhibition of the responses to MCP-3, markedly higher concentrations of the corresponding truncated analogs were required. At high concentration (1,000 nM), all truncated analogs also attenuated or even prevented the $[Ca^{2+}]$ changes induced by the other chemokines. RANTES (9-68) markedly decreased the $[Ca^{2+}]$ induced by MCP-3.

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EXAMPLE 5

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Three month old MRL-1pr mice, both male and female were injected at two thoracic sites with complete Freund's Adjuvant (CFA) only once on day 0.

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On that same day RANTES(9-68) was injected intraperitoneally. Injections of the RANTES analog were administered daily for 30 days at 2mg/kg/day.

The mice were visually examined by (a double-blind observation) every five days for appearance of arthritis. An animal was scored as positive for arthritis if erythema and swelling of a fore or hind paw was observed. In addition ankle width of the hind legs were assessed. Histopathology of the lime joints were evaluated on day 30 post-adjuvant injection.

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RESULTS:

Human RANTES(9-68) was able to bind to the receptor on mouse peritoneal macrophages. Native human RANTES did not bind well to mouse peritoneal macrophages. Human RANTES(9-68) bound to mouse peritoneal cells with an affinity (K_d) of 21 nM which is similar to the binding affinity of RANTES(9-68) to human monocytes (Figure 8).

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Daily i.p. injections of RANTES (9-68) inhibited the swelling and the incidence of arthritis in adjuvant treated mice. Treated mice exhibited insignificant swelling. In contrast, treatment with a control peptide had no inhibitory effect (Figures 9A and 9B).

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Histopathological studies demonstrated that
1 untreated control animals and animals administered a
peptide control developed rheumatoid arthritis,
substantial infiltration of mononuclear cells into the
synovial tissue, extensive hyperplasia of the synovial
5 lining, pannus formation and bone and cartilage
damage. In contrast, animals treated with RANTES(9-
68) exhibited no invasive pannus, no bone or cartilage
damage and insignificant inflammatory infiltration
(Figure 10).

10 Treatment with human RANTES(9-68) for 30
days was not toxic.

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EXAMPLE 6

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Three month old MRL-1pr mice, both male and female were injected at two thoracic sites with complete Freund's Adjuvant (CFA) only once on day 0. On that same day human MCP-3 antagonist MCP-3 (9-76) was injected intraperitoneally. Injections of the MCP-3 analog were administered daily for 30 days at 3 mg/kg/day. The mice were visually examined (by a double-blind observation) every five days for appearance of arthritis. An animal was scored as positive for arthritis if erythema and swelling of a fore or hind paw was observed. In addition ankle width of the hind legs was assessed. Histopathology of the limb joints were evaluated on day 30 post-adjuvant injection.

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RESULTS:

Human MCP-3 (9-76) was able to bind to the receptor on mouse monocytic cells (WEH1 265 cells). Human MCP-3 (9-76) did not bind well to mouse monocytes. Human MCP-3 (9-76) bound to mouse monocytic cells with an affinity (K_d) of 57 nM which is four-fold lower than that for native MCP-3 (K_d 225 nM) (Figure 11).

25

Daily i.p. injections of MCP-3 (9-76) inhibited the swelling and the incidence of arthritis in adjuvant treated mice. In contrast, treatment with a control peptide had no inhibiting effect (Figure

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12). Treatment with human MCP-3 (9-76) for 30 days
1 was not toxic.

Thus, in summary, in accordance with the
present invention, the inventors have discovered that
NH₂-terminally truncated analogs of native chemokines
5 lack chemoattractant and other activities. The
present inventors have also determined that the
analogs of the present invention competitively bind to
native chemokine receptors, thereby significantly
inhibiting or even precluding binding by the
10 corresponding native molecules. The inventors have
also discovered a correlation between the bound
analogs and their concomitant lack of biological
activity. It has now been discovered by the present
inventors that the analogs of the present invention
15 are useful in treating inflammatory diseases and
autoimmune diseases in mammals.

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WHAT IS CLAIMED IS:

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1. An analog of mammalian Monocyte Chemoattractant Protein-3 (MCP-3) lacking NH₂-terminal amino acids corresponding to amino acid residues 1-7,
5 1-8, 1-9 or 1-10 of MCP-3, having about at least 80% homology to said MCP-3 and having antagonist activity thereto.

2. The analog according to Claim 1, wherein
10 said analog has at least 85% homology to MCP-3.

3. The analog according to Claim 1, wherein said analog has at least 90% homology to MCP-3.

15 4. The analog according to Claim 1, wherein said analog has at least 95% homology to MCP-1.

5. The analog according to Claim 1, wherein said analog comprises MCP-3 (9-76) or a fragment
20 thereof.

6. An analog of mammalian Regulated on Activation Normal T Cell Expressed and Secreted protein (RANTES) lacking NH₂-terminal amino acids
25 corresponding to amino acid residues 1-5, 1-6, 1-7, 1-8, and 1-9 of RANTES, having about at least 60% homology to said RANTES and having antagonist activity to said RANTES.

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7. The analog according to Claim 6, wherein
1 said analog comprises RANTES (9-68) or a fragment
thereof.

8. The analog according to Claim 6, wherein
5 said analog comprises RANTES (6-68) or a fragment
thereof.

9. The analog according to Claim 6, wherein
said analog comprises RANTES (7-68) or a fragment
10 thereof.

10. The analog according to Claim 6,
wherein said analog comprises RANTES (8-68) or a
fragment thereof.

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11. The analog according to Claim 6,
wherein said analog comprises RANTES (10-68) or a
fragment thereof.

20 12. An analog of mammalian Macrophage
Inflammatory Protein-1 α (MIP-1 α) lacking NH₂-terminal
amino acids corresponding to amino acid residues 1-9
or 1-10, having about at least 60% homology to said
MIP-1 α and having antagonist activity to said MIP-1 α .

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13. The analog according to Claim 12,
wherein said analog comprises MIP-1 α (10-70) or a
fragment thereof.

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14. A method of inhibiting MCP-3 biological
1 activity in a mammal comprising administering to said
mammal a therapeutically effective amount of an analog
according to Claim 1, 6, or 12 for a time and under
conditions sufficient to inhibit said biological
5 activity.

15. A method of inhibiting RANTES
biological activity in mammals comprising
administering thereto a therapeutically effective
10 amount of an analog according to Claims 1, 6, or 12
for a time and under conditions sufficient to inhibit
said biological activity.

16. A method of inhibiting MIP-1 α
15 biological activity in mammals comprising
administering thereto a therapeutically effective
amount of an analog according to Claims 1, 6, or 12
for a time and under conditions sufficient to inhibit
said biological activity.

20 17. A method of treating an inflammatory
disease in a mammal comprising administering to a
mammal in need thereof a therapeutically effective
amount of an analog according to Claims 1, 6 or 12.

25 18. A method of treating an autoimmune
disorder in a mammal comprising administering to a
mammal in need thereof a therapeutically effective
amount of an analog according to Claims 1, 6 or 12.

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19. A method of treating rheumatoid
1 arthritis in a mammal comprising administering to a
mammal in need thereof a therapeutically effective
amount of an analog according to Claim 1, 6 or 12.

5 20. A pharmaceutical composition comprising
an antagonistically effective amount of an analog of
Claim 1 and a pharmaceutically acceptable carrier.

21. A pharmaceutical composition comprising
10 an antagonistically effective amount of an analog of
Claim 6 and a pharmaceutically acceptable carrier.

22. A pharmaceutical composition comprising
an antagonistically effective amount of an analog of
15 Claim 12 and a pharmaceutically acceptable carrier.

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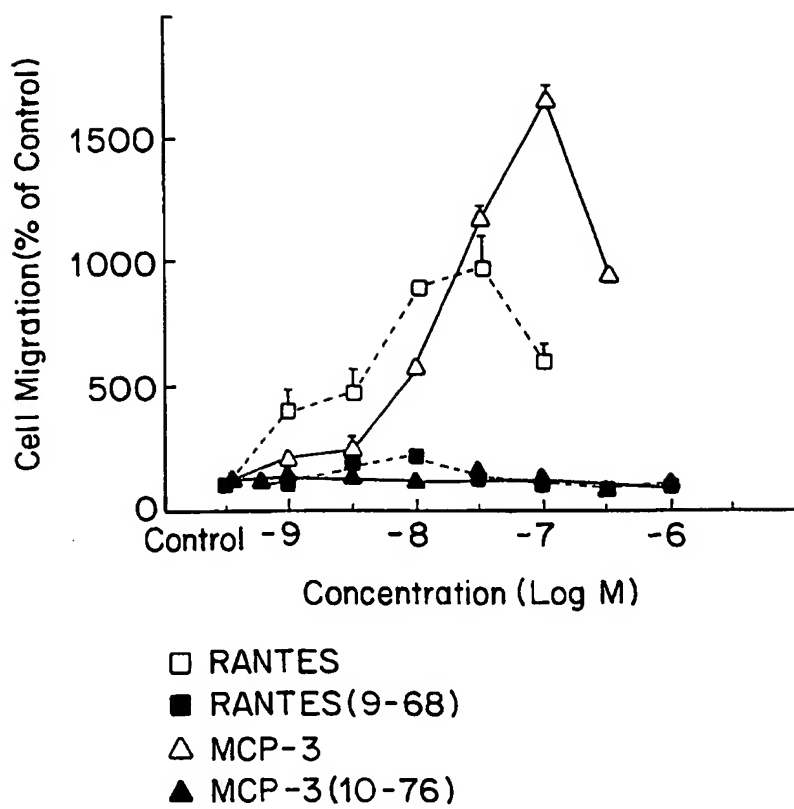
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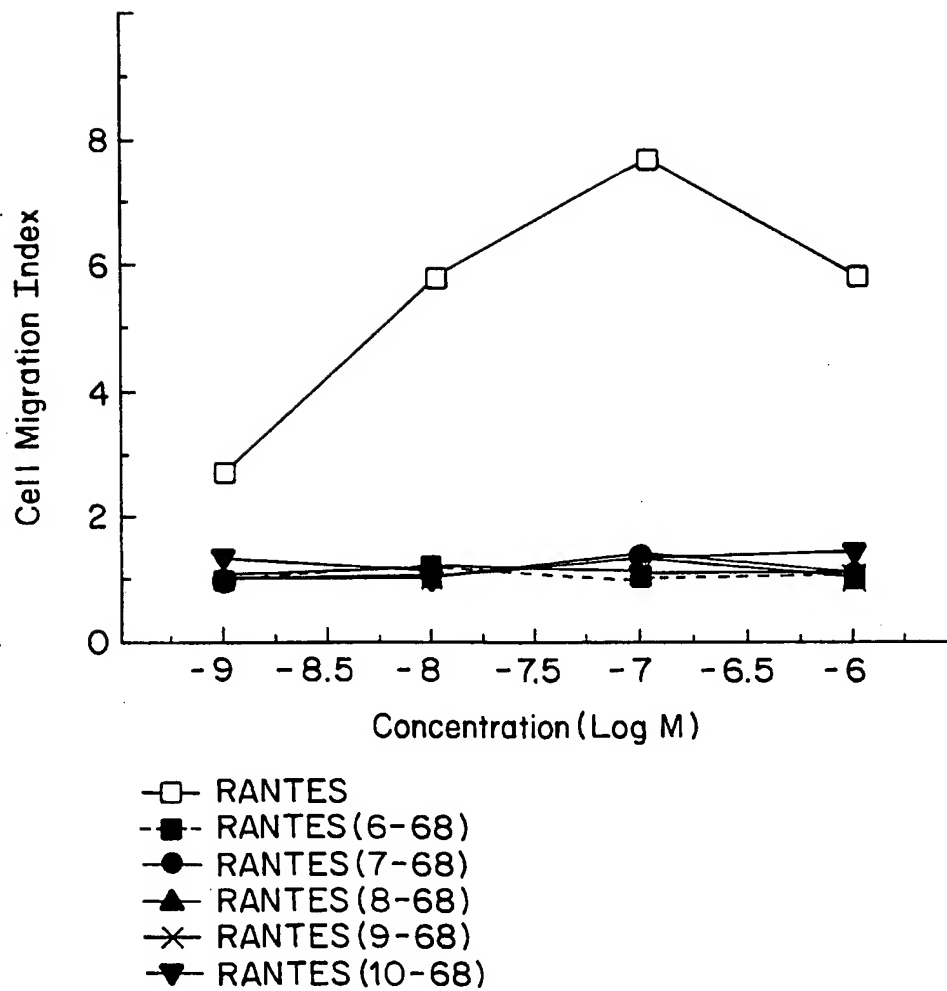
1 / 2 1

FIG. 1A



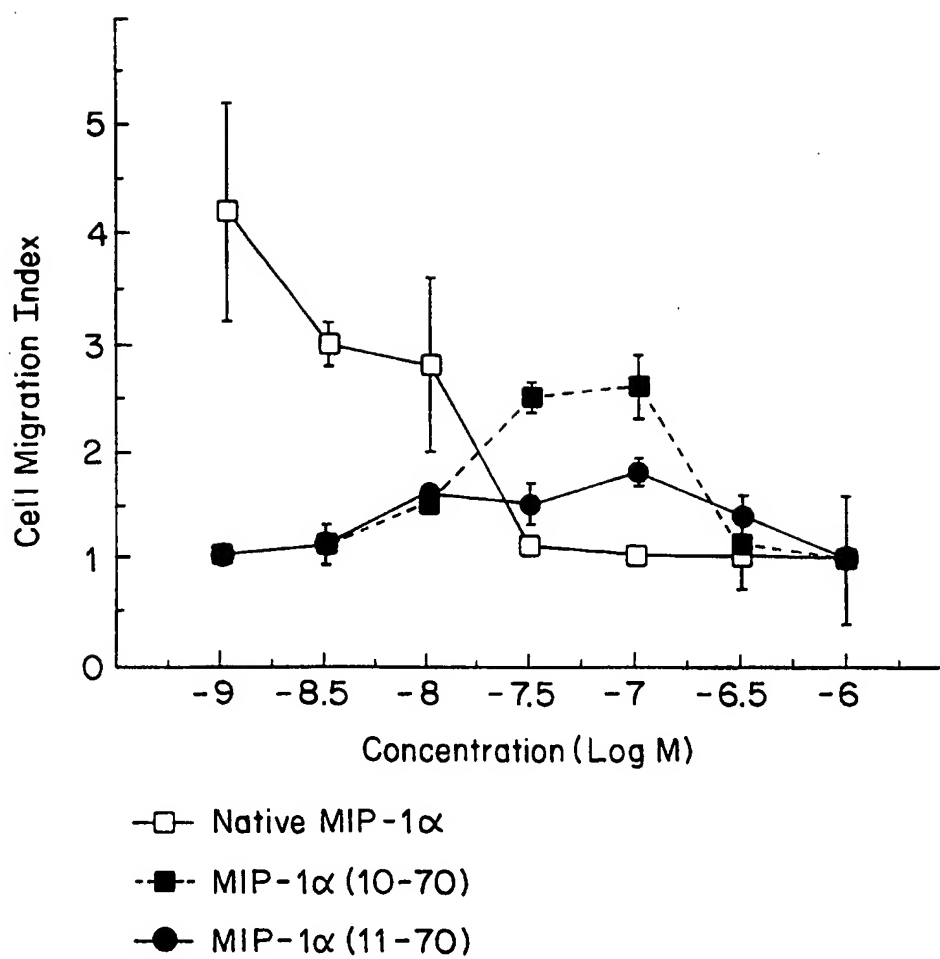
2 / 2 1

FIG. 1B



3 / 2 1

FIG. 1C



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FIG. 2A

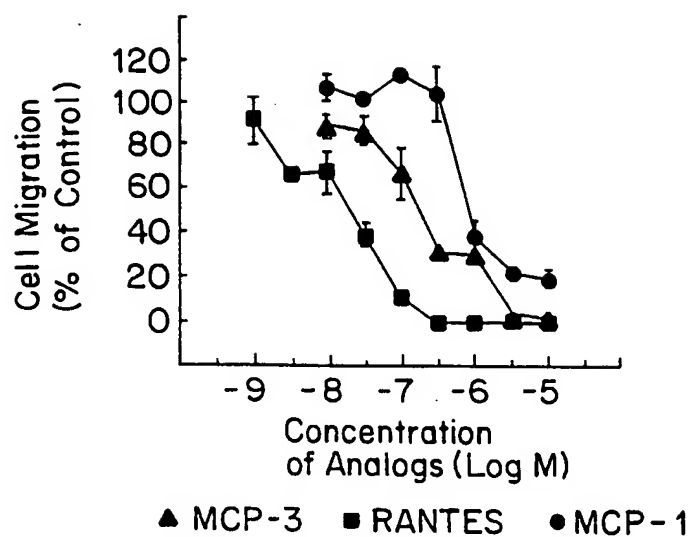
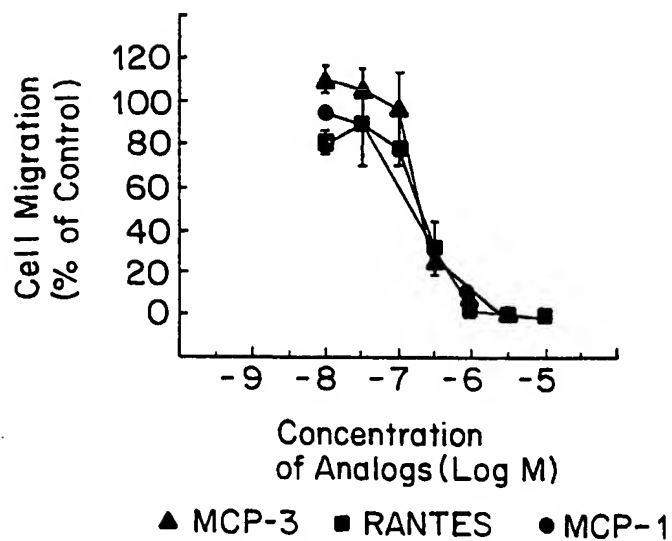
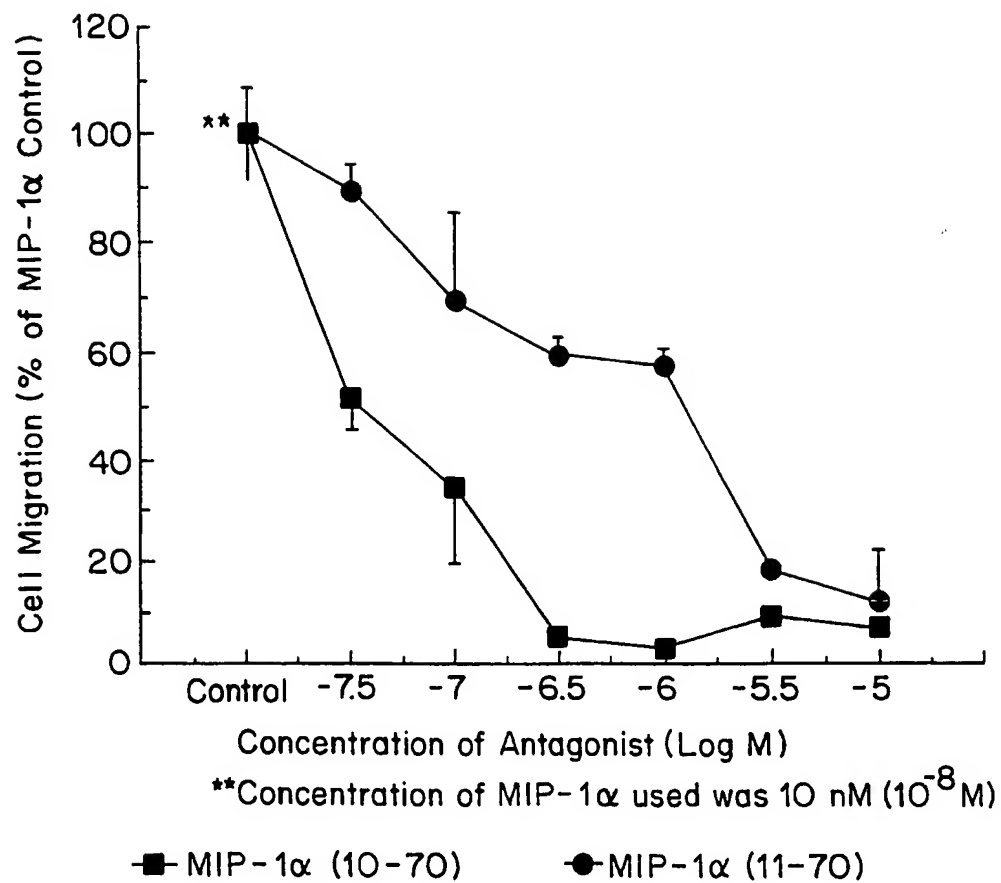


FIG. 2B



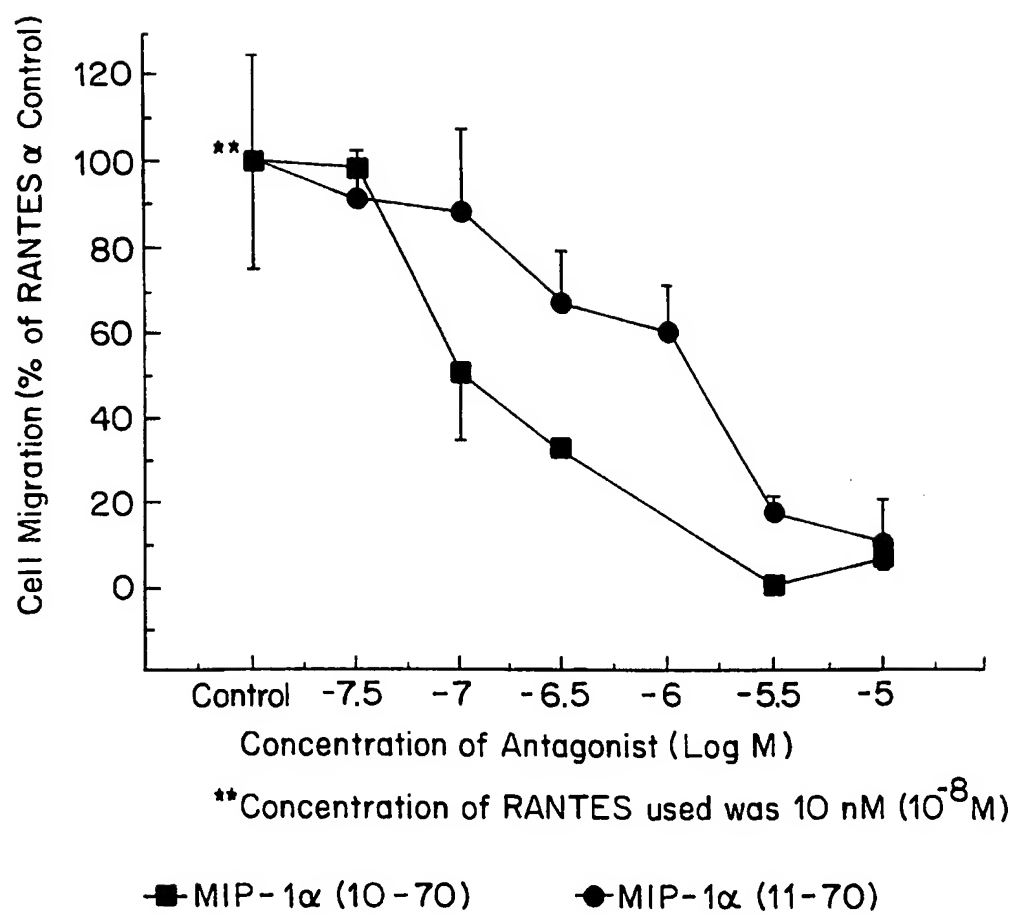
5 / 2 1

FIG. 2C



6 / 2 1

FIG. 2D



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FIG. 3A

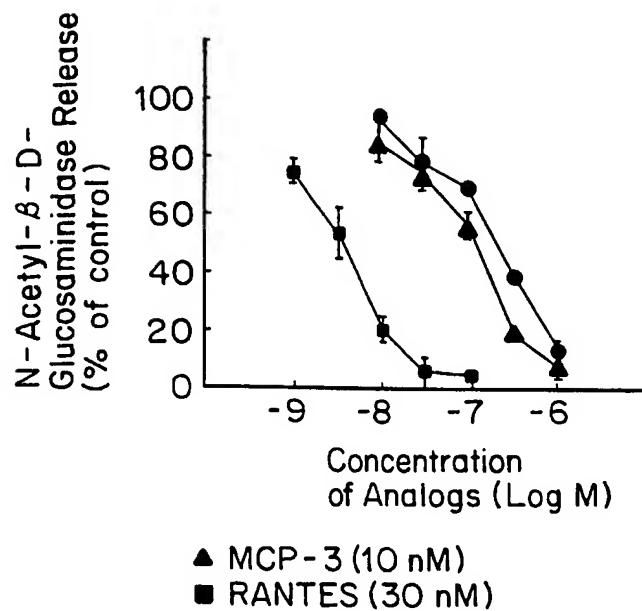
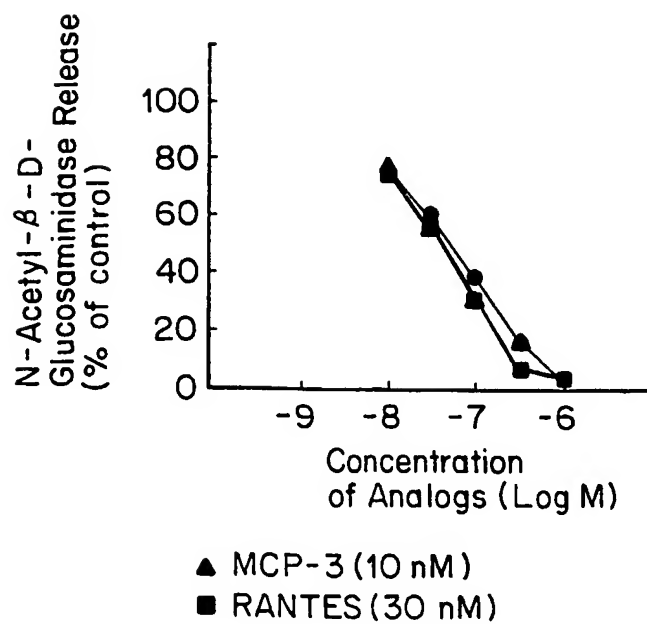


FIG. 3B



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FIG. 3C

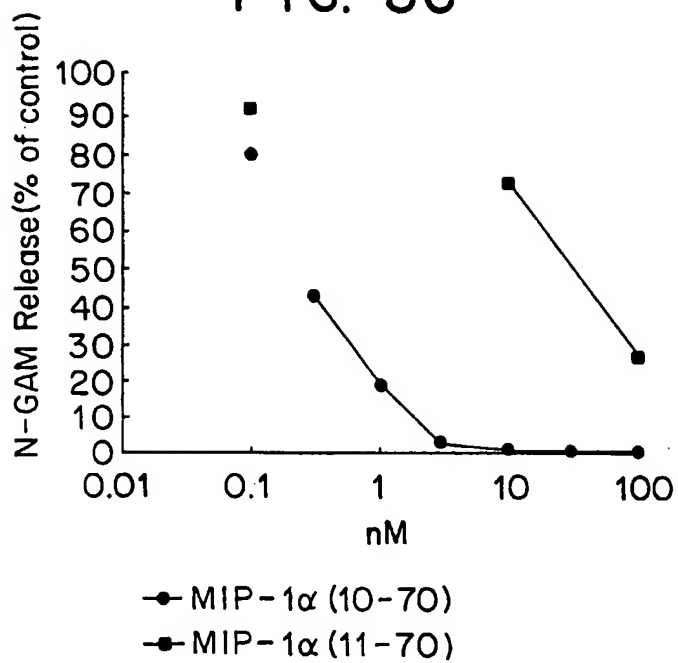
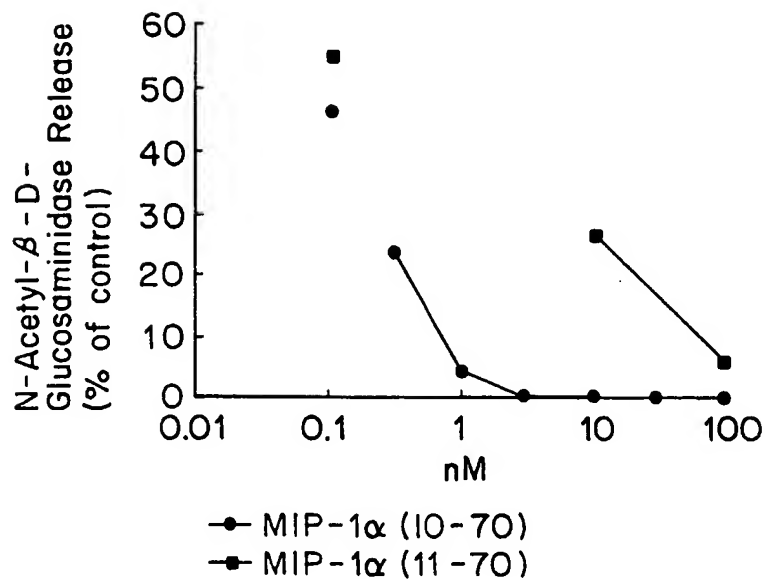


FIG. 3D



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FIG. 4A

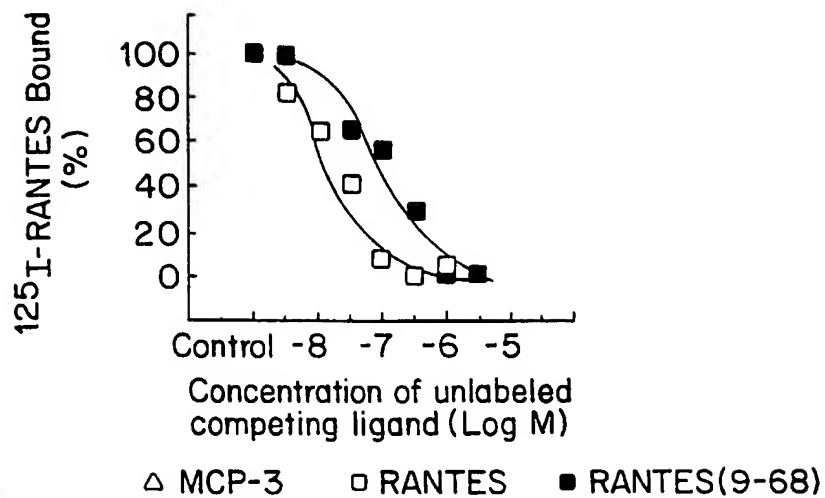
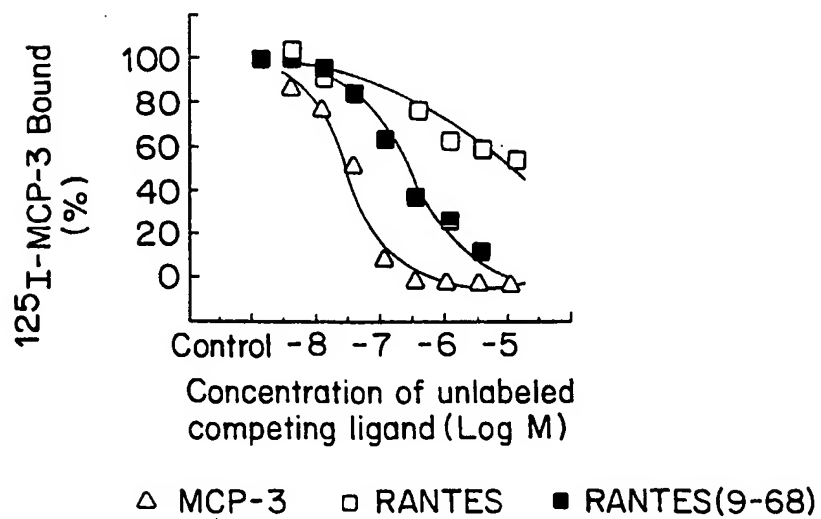


FIG. 4B



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FIG. 4C

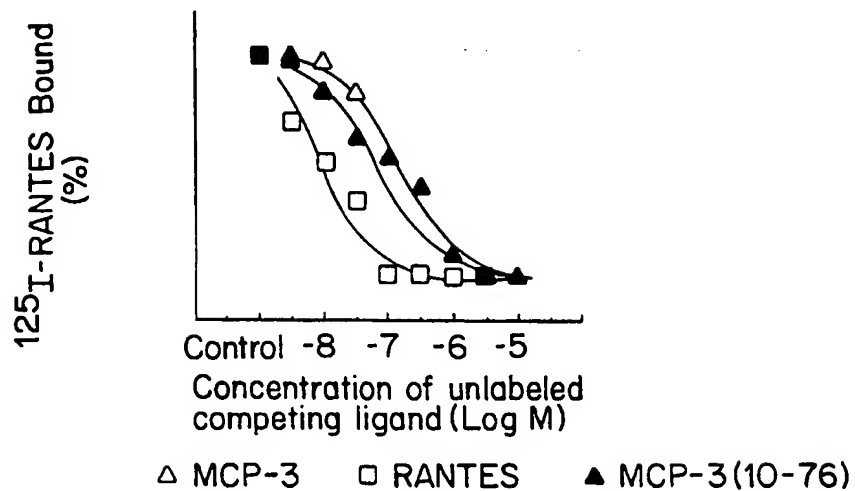
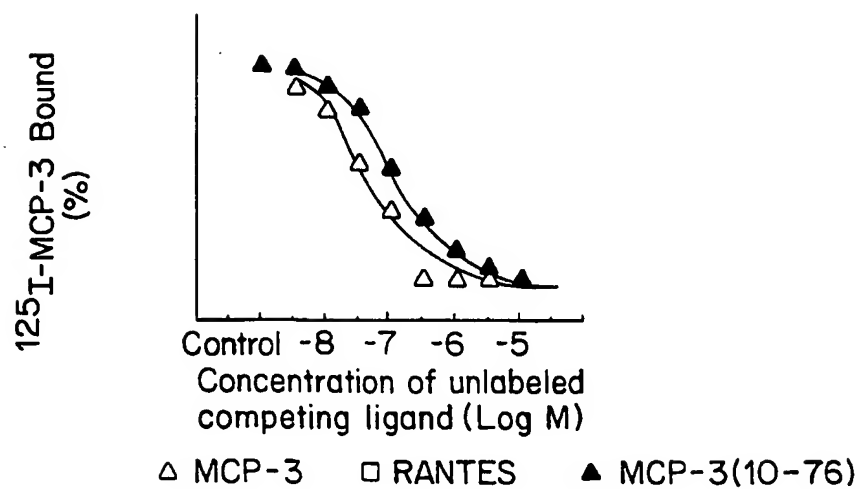
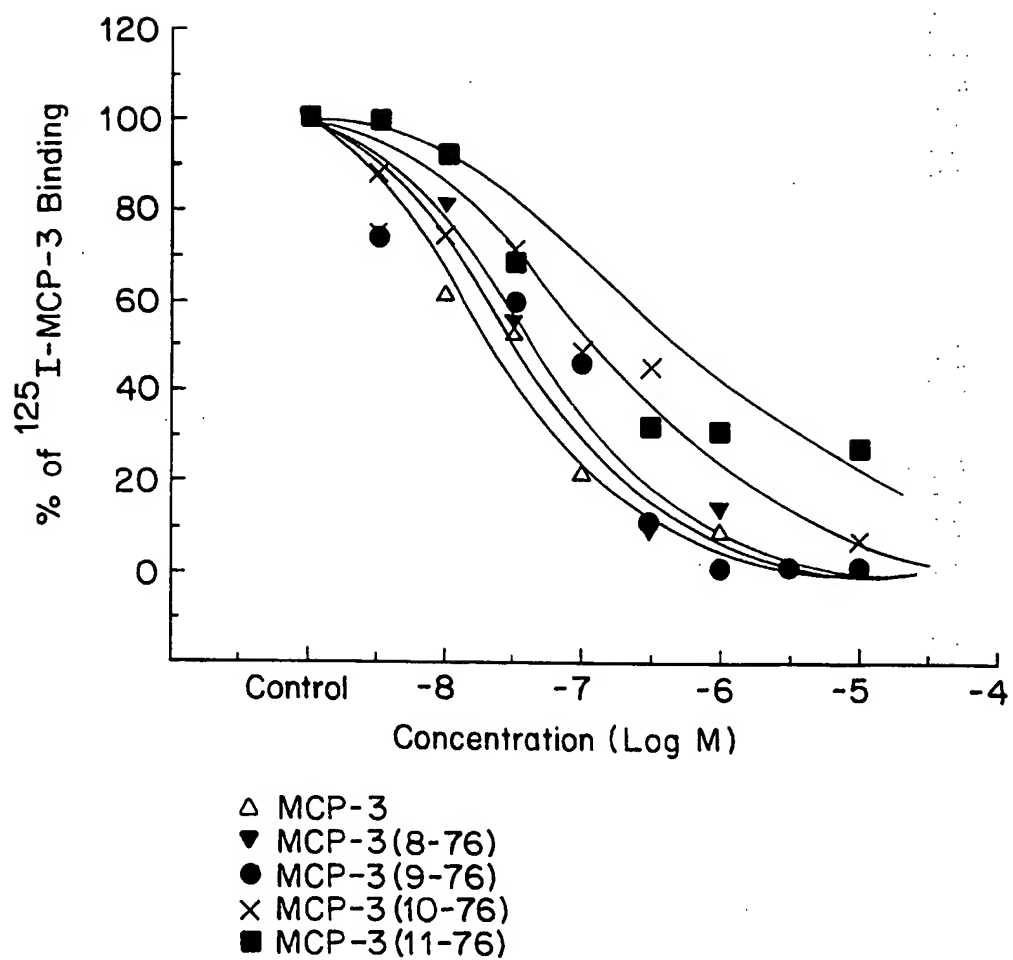


FIG. 4D



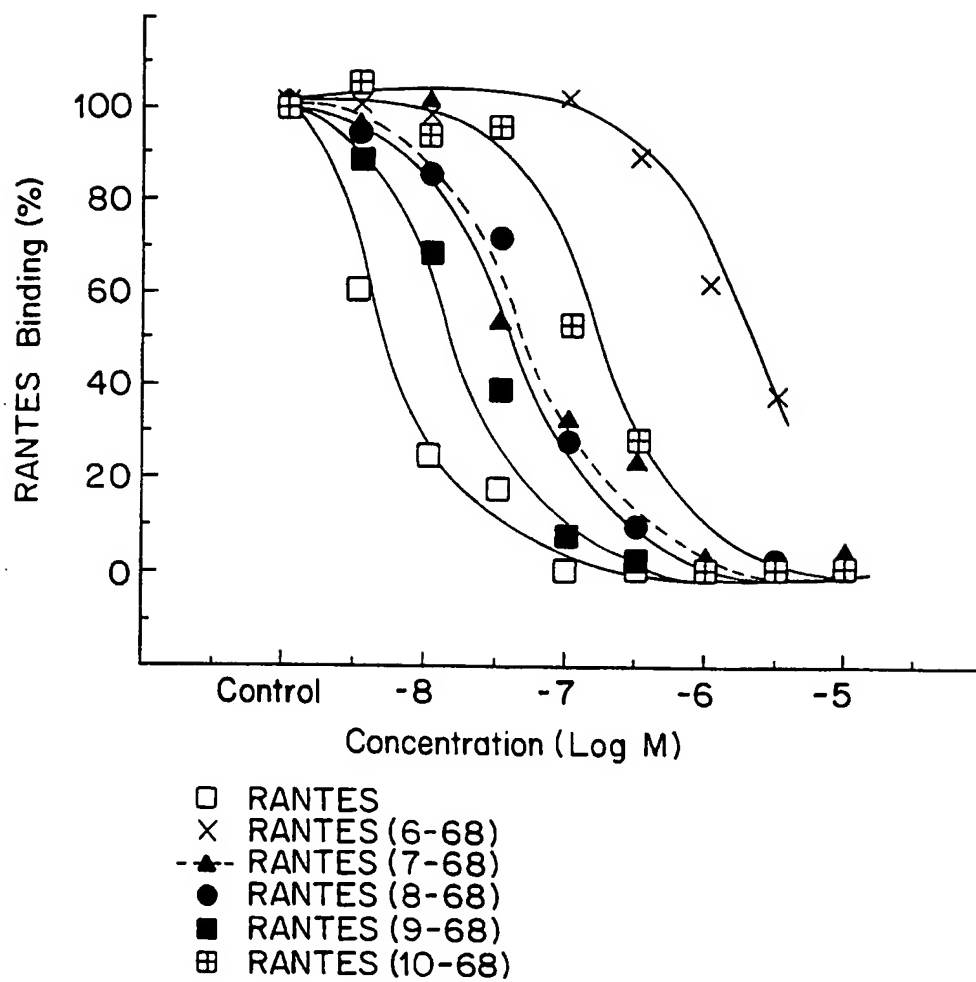
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FIG. 4E



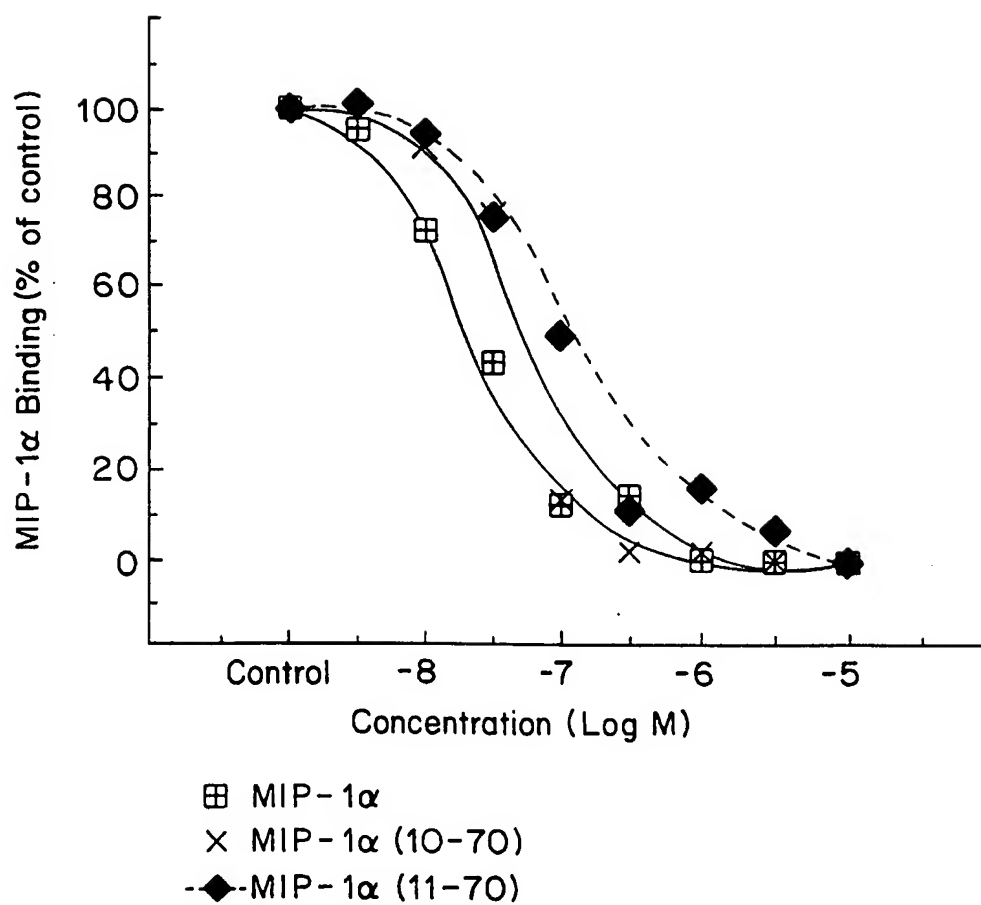
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FIG. 4F



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FIG. 5



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FIG. 6A

Chemokines or Antagonists	MIP-1 α	RANTES	MCP-3
<u>MIP-1α</u>			
Binding (Kd) *	5	10	437
MIP-1 α (10-70)			
Binding (Kd) *	25	14	281
Chemotaxis (IC ₅₀) **	56	100	800
N-Acetyl- β -Glucosaminidase Release (IC ₅₀) ***	1	1	>1,000
MIP-1 α (11-70)			
Binding (Kd) *	82	33	No Binding
Chemotaxis (IC ₅₀) **	560	794	>1,000
N-Acetyl- β -Glucosaminidase Release (IC ₅₀) ***	30	10	>1,000
RANTES (1-68)			
Binding (Kd) *		3 \pm 1	No Binding (Kd>500)

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FIG. 6B

RANTES (9-68)				
Binding (Kd)*	25	19		57
Chemotaxis (IC ₅₀)**	126	41		200
N-Acetyl-β-Glucosaminidase Release (IC ₅₀)***	20	4		170
MCP-3 (10-76)				
Chemotaxis (IC ₅₀)**		620 ± 330		470 ± 170
N-Acetyl-β-D-Glucosaminidase Release (IC ₅₀)***		48 ± 14		37 ± 5
Binding (Kd)*		50 ± 5		57 ± 5
MCP-3 (1-76)				
Binding (Kd)*		40 ± 9		28 ± 4

The indicated concentrations of chemokines and their antagonists were in nM.

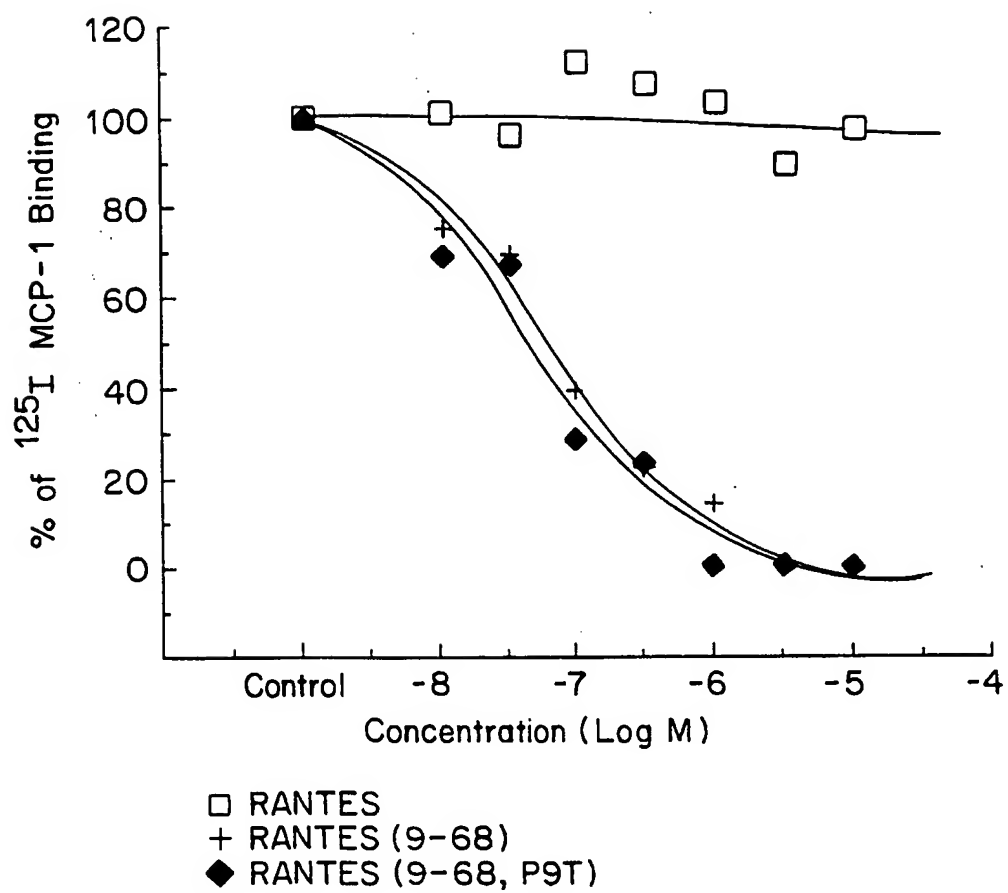
* The receptor binding study has been done on human monocytic cell line THP-1 cells. The concentration of the ¹²⁵I-labeled chemokines (MCP-3, RANTES or MIP-1α) was 4 nM.

** The Inhibition of chemotaxis was done on THP-1 cells. The concentration of all chemokines was 10 nM.

*** The inhibition of enzyme release (exocytosis function of monocytes) has been done on human peripheral monocytes. The concentration of RANTES and MIP-1α used for these experiments was 30 nM. The concentration of MCP-3 used was 10 nM.

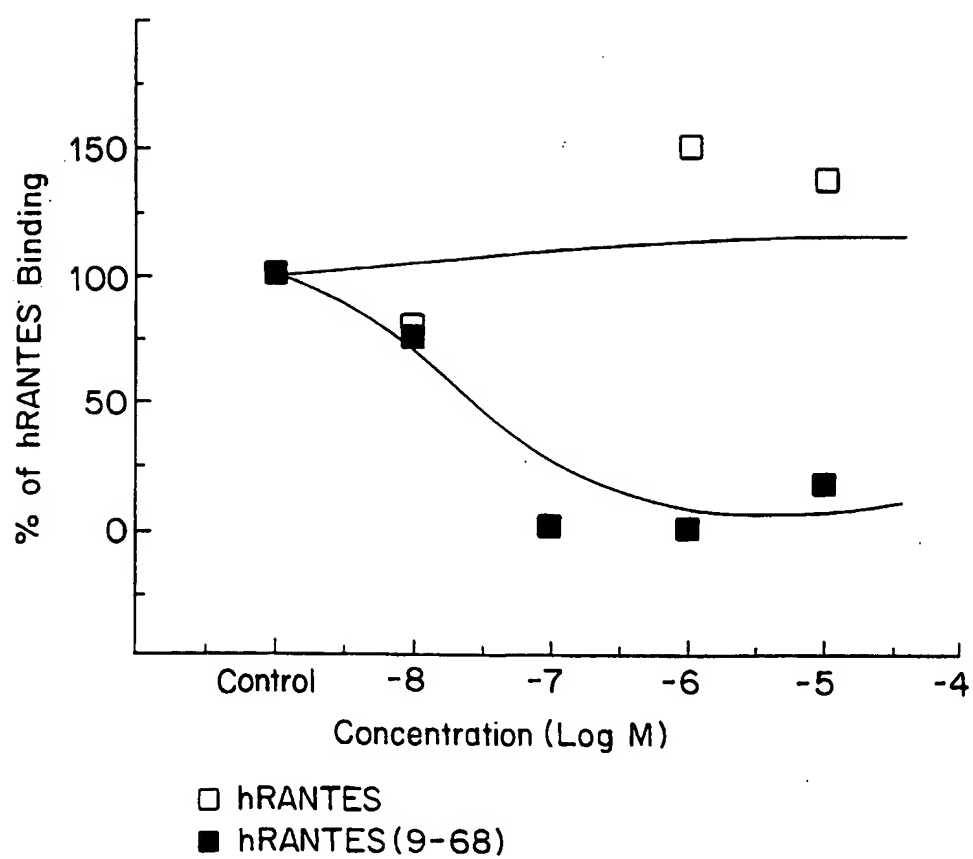
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FIG. 7



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FIG. 8



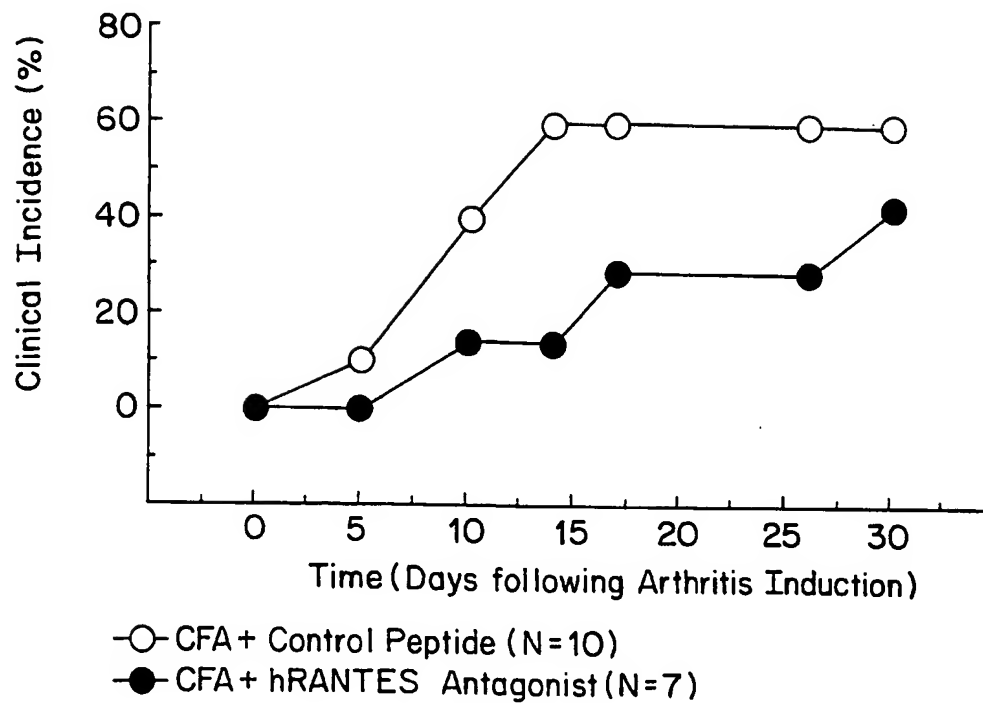
18/21
FIG. 9A

FIG. 9B

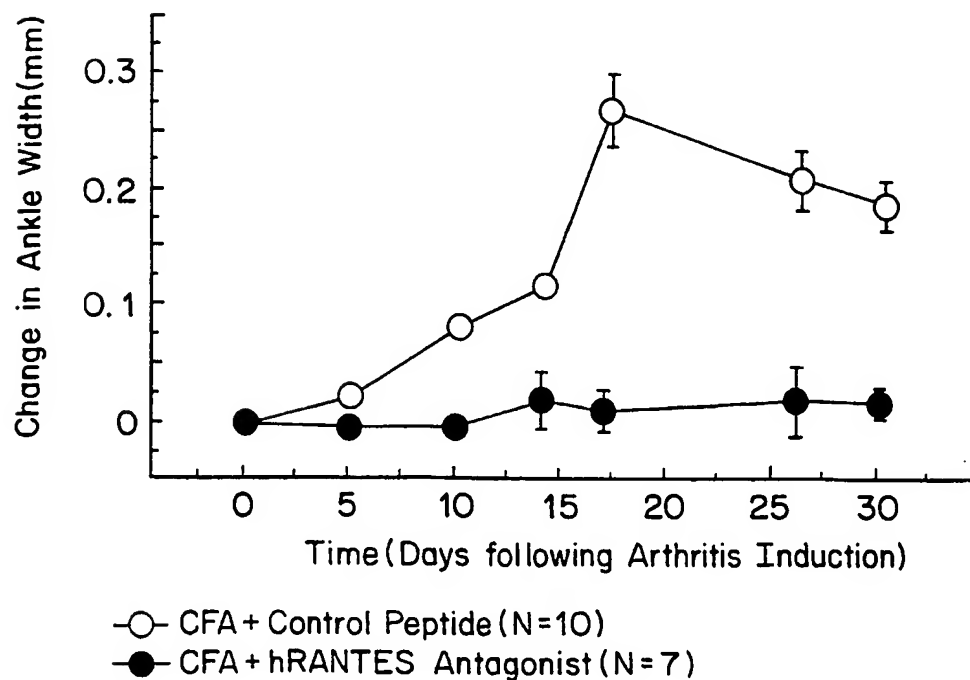
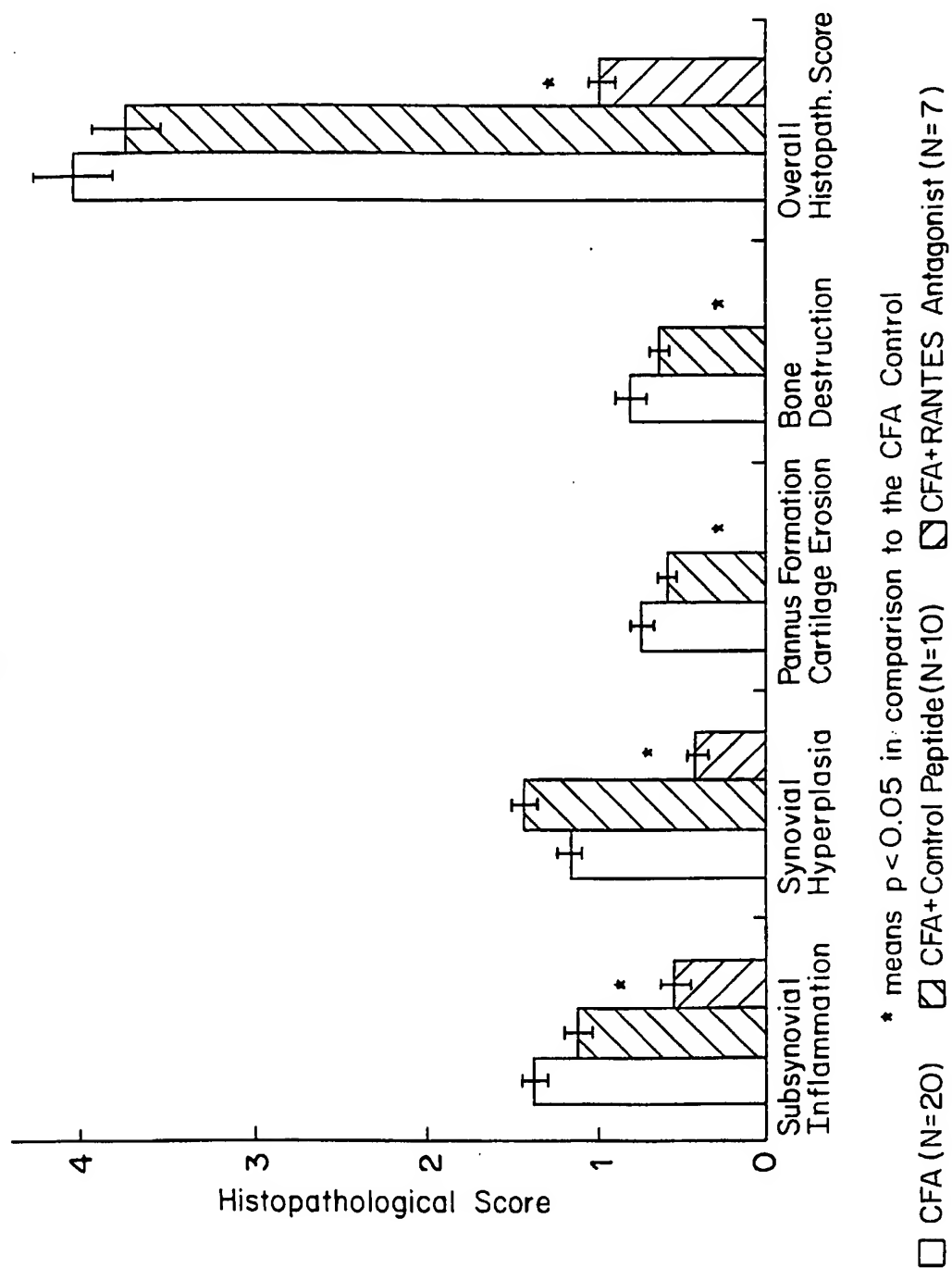
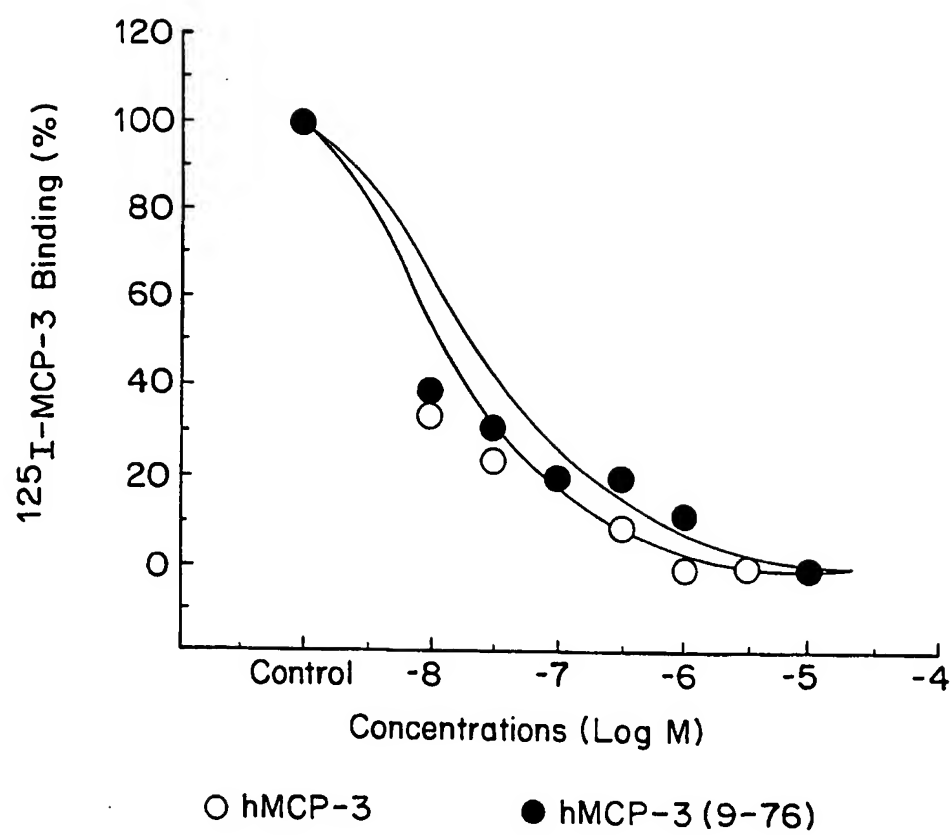


FIG. 10



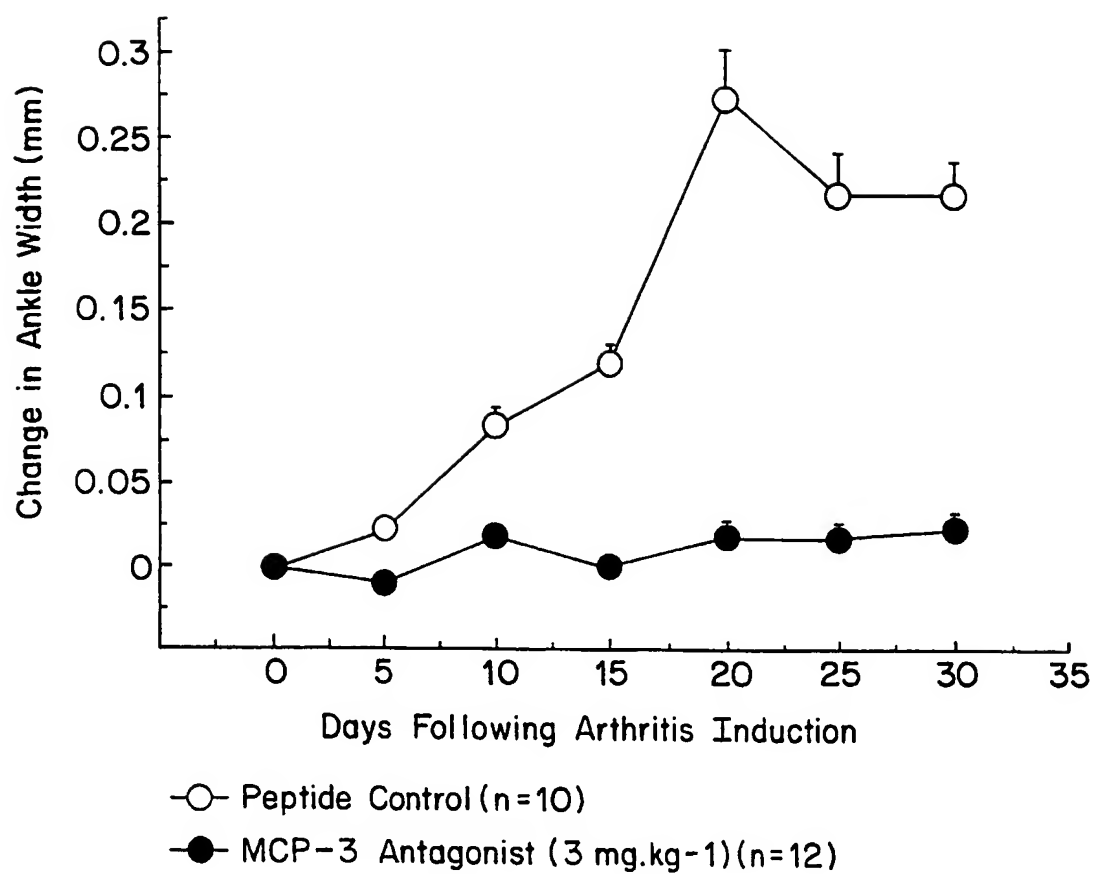
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FIG. II



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FIG. 12



INTERNATIONAL SEARCH REPORT

Intern. Appl. Application No

PCT/US 97/14485

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/52 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONG E.A.: "RANTES and MCP-3 antagonists bind multiple chemokine receptors" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 18, 3 May 1996, MD US, pages 10521-10527, XP002047804 see the whole document ---	1-22
X	CLARK-LEWIS I ET AL: "STRUCTURE-ACTIVITY RELATIONSHIPS OF CHEMOKINES" JOURNAL OF LEUKOCYTE BIOLOGY, vol. 57, no. 5, May 1995, pages 703-711, XP000605180 See especially pp.709-710 -----	1-22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

21 November 1997

Date of mailing of the international search report

12.12.97

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Groenendijk, M

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/14485

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 14-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INVITATION TO PAY ADDITIONAL FEES

International application No.

PCT/US 97/14485

1. Claims: 1-5,14,20(complete), 17-19(partially)

Truncated analogs of MCP-3 as defined in claim 1, their compositions and their use.

2. Claims: 6-11,15,21(complete), 17-19(partially)

Truncated analogs of RANTES as defined in claim 6, their compositions and their use.

3. Claims: 12,13,16,22(complete), 17-19(partially)

Truncated analogs of MIP-1alpha as defined in claim 12, their compositions and their use.